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<b>(21) International Application Number:</b> PCT/US96/06010 <b>(22) International Filing Date:</b> 29 April 1996 (29.04.96)  <b>(30) Priority Data:</b> 08/432,016 1 May 1995 (01.05.95) US  <b>(71) Applicants:</b> DUKE UNIVERSITY [US/US]; 230 North Building, Research Drive, Box 90083, Durham, NC 27708-0083 (US). BRISTOL-MYERS SQUIBB COMPANY [US/US]; 345 Park Avenue, New York, NY 10154 (US).  <b>(72) Inventors:</b> HAYNES, Barton, F.; 4923 Wentworth Drive, Durham, NC 27707 (US). ARUFFO, Alejandro; 1012 Spruce Street, Edmonds, WA 98020 (US). PATEL, Dhavalkumar; 3012 Deerpase Wynd, Durham, NC 27712 (US). BOWEN, Michael, A.; 200 Roy Street #540, Seattle, WA 98109-4100 (US). MARQUARDT, Hans; 9222 S.E. 46th Street, Mercer Island, WA 98040-4408 (US).  <b>(74) Agent:</b> WILSON, Mary, J.; Nixon & Vanderhye P.C., 8th floor, 1100 North Glebe Road, Arlington, VA 22201-4714 (US).		<b>(81) Designated States:</b> AU, CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> CD6 LIGAND  <b>(57) Abstract</b>  The present invention relates, in general, to CD6 and, in particular, to a CD6 ligand present on the surface of thymic epithelial cells, monocytes, activated T cells and a variety of other cell types. The invention further relates to methods of inhibiting the interaction of CD6 and the CD6 ligand, and to methods of screening compounds for their ability to inhibit that interaction.		

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### CD6 LIGAND

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5 certain rights in the invention.

This is a continuation-in-part of Application No. 08/333,350, filed November 2, 1994, which is a continuation-in-part of Application No. 08/143,903, filed November 2, 1993, the entirety of both  
10 applications being incorporated herein by reference.

### FIELD OF THE INVENTION

The present invention relates, in general, to CD6 and, in particular, to a CD6 ligand present on the surface of thymic epithelial cells, monocytes, activated  
15 T cells and a variety of other cell types. The invention also relates to a nucleic acid sequence encoding the CD6 ligand and to vectors and host cells comprising same. The invention further relates to methods of inhibiting the interaction of CD6 and the CD6  
20 ligand, and to methods of screening compounds for their ability to inhibit that interaction.

### BACKGROUND

CD6 is a 130 kDa glycoprotein that is homologous to CD5 and the macrophage scavenger receptor (Aruffo et al, J. Exp. Med. 174:949 (1991)). CD6 is expressed on the  
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surface of mature thymocytes, peripheral T cells and a subset of B cells (Kamoun et al, J. Immunol. 127:987 (1981)). CD6 appears to be involved in T cell activation since monoclonal antibodies (mabs) to CD6 augment T cell receptor (TCR) mediated activation of T cells (Gangemi et al, J. Immunol. 143:2439 (1989)), and the CD6 molecule is tyrosine phosphorylated during TCR-mediated T cell triggering (Wee et al, J. Exp. Med. 177:219 (1993)). Mabs to CD6 have been shown to be clinically useful for the deletion of T cells for allogeneic bone marrow transplantation (Soiffer et al, J. Clin. Oncol. 10:1191 (1992)).

Results obtained by screening a panel of mabs to T cell surface antigens for their ability to inhibit the binding of thymocytes to human thymic epithelial cells (TEC) suggested that CD6 is involved in the binding of thymocytes to TEC (Vollger et al, J. Immunol. 138:358 (1987)). The present invention relates to a ligand for CD6 involved in that binding and to a nucleic acid sequence encoding same.

#### OBJECTS AND SUMMARY OF THE INVENTION

It is the general object of the invention to provide a CD6 ligand.

It is a specific object of the invention to provide a CD6 ligand in isolated form and a nucleic acid sequence encoding that ligand.

It is a further object of the invention to provide a method of inhibiting the binding of CD6 to a CD6 ligand.

It is yet another object of the invention to provide a method of screening compounds for their ability to inhibit CD6/CD6 ligand binding.

5 In one embodiment, the present invention relates to an isolated CD6 ligand, including both divalent cation dependent and divalent cation independent forms thereof. The invention further relates to mimetopes of such a ligand.

10 In another embodiment, the present invention relates to a nucleic acid sequence encoding a CD6-ligand and to a vector and host cell comprising same. The invention also relates to a method of producing a CD6 ligand or a portion thereof that comprises culturing a host cell comprising a CD6 ligand encoding sequence or a  
15 portion thereof under conditions such that the sequence is expressed and the CD6 ligand or portion thereof is thereby produced.

In a further embodiment, the present invention relates to a method of inhibiting binding of CD6 present  
20 on the surface of a first cell to CD6 ligand present on the surface of a second cell. The method comprises contacting the CD6 present on the surface of the first cell with a soluble CD6 ligand, or mimetope thereof, under conditions such that the soluble CD6 ligand, or  
25 mimetope thereof, binds to the CD6 present on the surface of the first cell and thereby inhibits binding of the CD6 ligand present on the surface of the second cell to the CD6 present on the surface of the first cell. Alternatively, the method comprises contacting  
30 the CD6 ligand present on the surface of the second cell with soluble CD6, or mimetope thereof, under conditions

such that the soluble CD6, or mimetope thereof, binds to the CD6 ligand present on the surface of the second cell and thereby inhibits binding of the CD6 present on the surface of the first cell to the CD6 ligand present on the surface of the second cell.

5. In another embodiment, the present invention relates to a method of screening a test compound for the ability to inhibit binding of CD6 to CD6 ligand. The method comprises: i) contacting the test compound with one of CD6 and CD6 ligand under conditions such that a complex between the test compound and the CD6 or CD6 ligand can form, ii) contacting the combination of CD6 or CD6 ligand and test compound resulting from step (i) with the other of CD6 and CD6 ligand under conditions such that complexation between the CD6 and the CD6 ligand can occur, and iii) determining the extent of complexation between the CD6 and the CD6 ligand and thereby the ability of the test compound to inhibit binding of the CD6 to the CD6 ligand.

20 Further objects and advantages of the present invention will be clear from the description that follows.

#### BRIEF DESCRIPTION OF THE DRAWINGS

25 Figure 1. Effect of divalent cations on binding of CD6-Ig (= CD6-Rg) to thymic epithelial cells (TEC). TEC were incubated with 5  $\mu$ g of CD6-Ig or CD5-Ig in buffers containing 2% BSA, Tris-HCl buffered saline (TBS) and either 5 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$  or 5 mM  $\text{MnCl}_2$ , and assayed by indirect immunofluorescence and flow cytometry.

Shown is the relative binding of CD6-Ig in the various buffers. Relative binding was determined as the ratio of specific binding of CD6-Ig (fluorescence of CD6-Ig - fluorescence of CD5-Ig) in sample buffer to the specific binding in TBS. Shown is a representative of three experiments (except for TBS+Mg which was performed only once). Both  $\text{CaCl}_2$  and  $\text{MnCl}_2$  were able to significantly enhance the binding of CD6-Ig to TEC ( $p \leq 0.1$ ).

Figure 2. CD6-Ig binding to TEC is enhanced by  $\text{Mn}^{++}$ . TEC were incubated with 5  $\mu\text{g}$  of CD6-Ig or CD5-Ig in buffers containing 2% BSA, TBS and various concentrations of  $\text{MnCl}_2$ , and assayed by indirect immunofluorescence and flow cytometry. The graph shows the relative binding of CD6-Ig in buffers containing varying amounts of  $\text{MnCl}_2$ . Shown is a representative of three separate experiments.

Figure 3. Binding to TEC is enhanced by the expression of CD6 in COS cells. COS cells that express CD6 in a stable manner (COS-CD6D) or control COS cells (COS-Neo) were incubated with calcein-AM labelled TEC at a ratio of 1:5 for 60 min at 4°C. Binding was detected by using a combination of light and fluorescence microscopy. Binding was determined to be the percentage of COS cells with 3 or more bound TEC. The expression of CD6 in COS cells enhanced the binding of COS cells to TEC, and this binding was inhibited by anti-CD6 antibody.

Figure 4. Monoclonal antibody J4-81 inhibits the binding of human CD6-Ig fusion protein to human TEC. The binding panel of 470 mabs from the V International Workshop on Human Leukocyte Differentiation Antigens and a panel of anti-integrin mabs was screened for reactivity to TE cells. Of the 154 mabs that reacted with TE cells, 126 were used in assays to inhibit the binding of CD6-Ig to TE cells. The workshop mabs that reacted with TE cells and were used in this study were:

5. A008, A014, A023-4, A036-7, A044, A047-9, A063, A065, A070, A074, A080, A090, A092, A107, A110, A113, A124, A132, A139, A141, A145, B002, B003, B005-6, B011-2, B014, B019, B025, B027-9, B031, B046, B049, B052, B055-7, B059, B068, CB10, CB22, CB27, E001, E015, E031, E045, E050, E053-4, E056-7, M01-2, M09, M14-5, M25, M32, M35-8, M43-4, MR1, MR4, MR6, MR9, MR12, MR14, XB001, NK32, P005, P012, P023, P036, P044, P098, P107-8, S009, S013, S023-4, S031, S075, S107, S188, S201, S241, S245, S252, S263, S271, S273-4, 5T-015, 5T-076, 5T-080, 5T-084, CD24.3, CD40.2, CD73.1, CD74.1 and CD77.1. Anti-integrin mabs used were: TS2/7, P1H6, 12F1, P1B5, P4G9, B5610, P3010, EA-1-6, 135-13C, P502, P309, K20, B4, P3G8, P3G2, P1H12 and TS2/16. The cells were pre-incubated with mabs at a dilution of 1:100 for 15 min at 4°C followed by incubation with 5 µg of either CD5-Ig or CD6-Ig. Cells were washed and fusion proteins detected by reaction with fluorescein conjugated antiserum specific to the Fc portion of human IgG and flow cytometry. Specific binding was determined to be the difference in fluorescence of CD6-Ig binding and CD5-Ig binding. The binding compared to control mab (P3) is

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shown. There were four instances where goat anti-human IgG-Fc cross reacted with the test mab (A024, B049, 5T-084 and CD73.1). Mab J4-81 (S252) inhibited CD6-Ig binding by  $56 \pm 5\%$  (N=2).

5           Figure 5. Mab J4-81 reacts strongly with the surface of all cultured TEC. Cultured TEC were incubated with mab J4-81 (S252 from the Vth International Workshop on Human Leukocyte  
10           Differentiation Antigens) and control mab (P3) for 30 min at 4°C. Following a wash with PBS containing 2% BSA, bound antibodies were detected by reaction with fluorescein conjugated goat anti-mouse IgG followed by flow cytometry. Shown are the fluorescence profiles of control mab (P3) and the mab against a CD6 ligand  
15           (J4-81).

          Figure 6. Expression of CD6 and its ligand in postnatal human thymus. CD6 and its ligand in frozen human thymus sections (4  $\mu$ m) were labelled by indirect immunofluorescence using mabs T12 and S252 (J4-81),  
20           respectively. Panel 1 shows the pattern of reactivity to T12 and panel 2 shows the pattern of reactivity to mab J4-81. At least three different thymus tissues were analyzed with similar results. CD6 was expressed on thymocytes in the medulla (med) and moderately on  
25           thymocytes in the cortex (ctx). Mab J4-81 detected medullary thymic epithelial cells and Hassal's bodies (HB).

Figure 7. Immunoprecipitation of radioactively labelled glycoproteins from metabolically labelled TEC with CD6-Ig. Cells were labelled with 6-<sup>3</sup>H-glucosamine for 48 hours, lysed and lysates were immunoprecipitated with CD6-Ig and with CTLA4-Ig as a control. CD6-Ig immunoprecipitation of TEC lysates was carried out with and without inclusion of 15 mM EDTA. The immunoprecipitation of a 105 kDa molecule and a 35 kDa molecule by CD6-Ig was divalent cation independent and the immunoprecipitation of an additional 90 kDa molecule by CD6-Ig was divalent cation dependent.

Figure 8. CD6-Rg (= CD6-Ig) binds to a number of human-derived cell lines. Binding of CD6-Rg (solid line) and CD5-Rg (dashed line) to a number of human cell lines was examined by flow cytometry. The cell lines were grouped into three categories based on their ability to bind CD6-Rg high binding, low binding, and no binding. The percentage binding used to group cells into these three categories is based on the number of cells whose fluorescence intensity is greater than that of cells stained with the isotype matched CD5-Rg fusion protein (vertical dash-dot line). The human cell lines examined were: (a) HBL-100, (b) H3719, (c) H3606, (d) LCL8664, (e) GM0833, (f) IMR90, (g) Jurkat, (h) Peer, (i) HUT78, (j) HPB-ALL, (k) JM, (l) H9, (m) LTR228, and (n) Raji. A total of 10<sup>4</sup> cells were analyzed in each experiment.

Figure 9. CD6-Rg binding is saturable and trypsin sensitive. (A) The binding of increasing concentrations

of CD6-Rg and CD5-Rg to the breast carcinoma cell line HBL-100 was examined by flow cytometry. The percentage binding (ordinate) was determined as described in Figure 8. A total of  $10^4$  cells were examined at each protein concentration. (B) The binding of CD6-Rg to HBL-100 cells which were pretreated with trypsin was compared to the binding of CD6-Rg and CD5-Rg to untreated HBL-100 cells by flow cytometry. This figure shows the results of a representative experiment out of three. Trypsin digests were carried out as described.

Figure 10. CD6-Rg binding is divalent cation dependent. The binding of CD6-Rg to HBL-100 cells in the presence of EDTA (+EDTA, dotted line) was compared to the binding of CD6-Rg (dash-dot line) and CD5-Rg (dashed line) by flow cytometry. The effect of EDTA on CD6-Rg binding to HBL-100 cells was also examined after the CD6-Rg/HBL-100 complexes were allowed to form (+EDTA, solid line). A total of  $10^4$  cells were examined in each experiment.

Figure 11. CD6-Rg binding is blocked by an anti-CD6 mAb. The ability of two different anti-CD6 mAb (MBG6 and G3-6) and an irrelevant IgM mAb to block the binding of CD6-Rg to HBL-100 cells was examined by flow cytometry. In each case serial dilution of the antibodies was tested.

Figure 12. CD6-Rg binding is modulated by cytokines. The binding of CD6-Rg (solid line) and CD5-Rg (dashed line) to untreated HBL-100 cells or HBL-100

cells treated with the indicated cytokines was examined by flow cytometry. The percentage of HBL-100 cells whose fluorescence intensity is to the right of an arbitrary marker (dash-dot line) following CD6-Rg binding is noted in each panel. The data shown in columns (A) and (B) represent two different experiments.

Figure 13. Immunoprecipitation of CD6-Rg binding protein(s). HBL-100 cells were radiolabeled with [<sup>3</sup>H]glucosamine. Protein(s) reactive with either CD6-Rg CTLA4-Ig, or an anti-EGF receptor mAb were immunoprecipitated from cell lysates in the absence or presence of EDTA and analyzed by SDS-PAGE. The arrows point to the two radiolabeled polypeptides which bound to CD6-Rg in the absence of EDTA. Molecular masses are given in kDa.

Figure 14. CD6-Rg binds to cells in lymphoid organs. (A) CD6-Rg binding to skin. Scattered cells in the dermis are labeled. Nonspecific labeling of hair shafts is indicated by arrows. (B) Reactivity of human IgG1 with a serial skin section. Only weak labeling of hair shafts is evident (arrows). (C) Reactivity of CD6-Rg with thymus tissue. A delicate reticular labeling pattern is present in the cortex. (D) Reactivity of human IgG1 with thymus tissue. Only faint background labeling was detected. (E) Reactivity of CD6-Rg with lymph node. Cells in the intermediate or paracortical sinuses were labeled. (F) No binding of human IgG1 to lymph node tissue was observed.

Figure 15. Human epidermal keratinocytes express a surface ligand(s) for CD6. Shown are the reactivities of cultured epidermal keratinocytes with CD6-Rg, CD5-Rg, or ELAM1-Rg. Cells were incubated with 5  $\mu$ g of fusion protein in PBS containing 2% BSA and washed. The fusion proteins were labeled with fluorescein conjugated goat antiserum to human IgG and assayed by flow cytometry.

Figure 16. CD6-CD6 ligand interactions mediate TE-thymocyte binding. Shown is a summary of 5 separate TE-thymocyte binding experiments. Panel A depicts the amount of rosette formation in the presence of specific antibodies and panel B depicts the amount of rosette formation in the presence of fusion proteins. Standard errors are indicated by bars. Both anti-CD6 antibody and CD6-Rg fusion protein partially inhibit TE-thymocyte binding: T12 inhibited rosette formation by  $49 \pm 9\%$  and CD6-Rg inhibited rosette formation by  $35 \pm 9\%$ . The differences in binding between control and 35.1 ( $p < 0.003$ ), T12 ( $p < 0.015$ ) and CD6-Rg ( $p < 0.05$ ) were statistically significant.

Figure 17. Binding of COS-CD6 to TE cells is CD6-specific. Shown are histograms of CD6 expression on COS-neo (control) cells and COS-CD6 cells as determined by indirect immunofluorescence with mAb T12 and flow cytometry. Background fluorescence (control mAb P3) is depicted by a dotted line and CD6 expression is indicated by a solid line. The bar graph depicts COS-neo and COS-CD6 binding to TE cells in the presence of

either mAbs P3 or T12. Shown are the mean and SEM of three separate experiments.

Figure 18. Expression of CD6 on thymocyte subsets. Thymocytes were stained with mAb T12, fluorescein conjugated Fab fragments of goat anti-mouse IgG and a combination of CD4-PE and CD8-cychrome and analyzed on a FACStar<sup>Plus</sup> flow cytometer. Shown are data representative of experiments on 3 different thymuses with histograms of CD6 expression on all thymocytes, CD4+CD8+ (immature, DP) thymocytes, CD4+CD8- (mature, SP4) and CD4-CD8+ (mature, SP8) thymocytes. Background fluorescence (with control mAb P3) is shown in each histogram.

Figure 19. CD6 mediates the binding of both mature and immature thymocytes to TE cells. CD1+ or CD1- thymocytes were incubated with TE cells in the presence of either control antibody (P3), anti-CD2 (35.1) or anti-CD6 (T12) and assayed for rosette formation. The average percentage inhibition of TE-thymocyte binding and SEM (relative to control antibody) by anti-CD2 and anti-CD6, from 3 separate experiments, is shown. Inhibition of CD1+ (immature) thymocyte binding to TE cells is depicted by open boxes and inhibition of CD1- (mature) thymocyte binding is depicted by filled boxes. The binding of both the mature and immature subsets of thymocytes to TEC is inhibited by anti-CD2 and anti-CD6 antibodies. The difference in inhibition of CD1+ vs. CD1- cells by anti-CD2 mAb 35.1 was statistically

significant ( $p < 0.01$ ) but the difference in inhibition by anti-CD6 mAb T12 was not ( $p < 0.25$ ).

Figure 20. Thymic epithelial cells and thymic fibroblasts express a trypsin-sensitive surface ligand for CD6. (A) Shown are representative histograms depicting the reactivities with CD6-Rg of thymic epithelial cells, thymic fibroblasts, and thymocytes. Also shown in each panel are background levels of fluorescence with control fusion protein CD5-Rg. (B) The binding of CD6-Rg to TE cells in either divalent cation containing media (- EDTA), trypsin-treated TE cells in media (trypsin) or TE cells in PBS + 10 mM EDTA (EDTA) was tested. Shown are histograms of a representative experiment (from >10 performed) depicting CD6-Rg binding to TE cells as determined by indirect IF and flow cytometry.

Figure 21. Effect of preincubation of J4-81 and J3-119 on CD6-Rg binding to TE cells. Shown are histograms of CD6-Rg binding to the surface of human TE cells, as detected by indirect IF and flow cytometry, in the presence of mAbs P3 (control), J4-81 or J3-119 in media containing divalent cations (- EDTA) or no divalent cations (+ EDTA). The binding of control human IgG is shown in each panel. The bottom panels show composite summary histograms of CD6-Rg binding in the presence of either P3, J4-81 or J3-119. Data are representative of 10 experiments in the presence of divalent cations and 2 experiments with no divalent cations.

Figure 22. MAb J3-119 blocks the binding of biotinylated J4-81 to the surface of TE cells. Shown is the specific binding of biotinylated J4-81 (in fluorescence units minus background) in the presence of increasing concentrations of mAbs A3D8, J3-119 and J4-81. Diluted ascites (1:50, 1:100, 1:250 and 1:500) was used and relative amount of mAb is presented as the inverse of the dilution  $\times 10^3$ . MAb A3D8, which binds strongly to the surface of TE cells did not alter the binding of J4-81 while both J3-119 and non-biotinylated J4-81 inhibited the binding of biotinylated J4-81. Data are representative of 2 experiments.

Figure 23. MAb J4-81 and CD6-Rg bind to the same 100 kDa glycoprotein on the surface of human TE cells. Shown are autoradiographs of  $^{125}\text{I}$ -labelled TE cell surface proteins cross-linked to either mAb P3 (control), mAb J4-81, hIgG1 (human Fc control), or CD6-Rg. The cross-linked proteins were treated with either 2-ME alone (to cleave the cross-linker) or trypsin and 2-ME prior to electrophoresis. Both CD6-Rg and mAb J4-81 bound to 100 kDa proteins with identical trypsin digestion patterns. Data are representative of 2 separate experiments.

Figure 24. Expression of CD6L-100 or ALCAM in postnatal human thymus. Shown are photomicrographs (representative of experiments on 5 thymuses) of frozen sections of 2 month human thymus stained by indirect IF with mAb J4-81. Panel A shows a section of thymus stained by indirect IF with mAb J4-81. Panel A shows a



section of thymus cortex and panel B shows a section of thymus medulla with Hassall's bodies (HB). The thymic capsule is indicated by dashed lines in panel A. Thymic epithelial cells (arrows), in both the cortex and in and around HB in medulla, reacted with mAb J4-81. Thymocytes did not react with mAb J4-81. An identical pattern was seen using mAb J3-119 (not shown) (400X magnification).

Figure 25. Binding of COS-CD6 to TE cells is CD6-specific. Fig. 25A. Histograms of CD6 expression on COS-neo (control) and COS-CD6 cells were determined by indirect immunofluorescence with the anti-CD6 mAb T12 (Gangemi et al, J. Immunol. 143:2439-2447 (1989)) (solid line) or a control antibody P3 (dotted line). Fig. 25B. The bar graph depicts COS-neo and COS-CD6 binding to TE cells in the presence of either the T12, J4-81 or P3 mAbs. Shown are the mean and SEM of three separate experiments.

Figure 26. Sequence analysis of the predicted amino acid sequence of the CD6 ligand designated ALCAM, Northern analysis of ALCAM mRNA expression, and ALCAM cell surface expression by activated T cells. Fig. 26A. Alignment of the immunoglobulin-like extracellular domains of ALCAM (residues 35-512), BEN, neurolin, RAGE and MUC18. The lower case letter in front of the protein name designates the species (human, h, chicken, c, and fish, f). Consensus residues are those shared by three or more proteins. Invariant residues are shown shaded and Cys's are highlighted with an asterisk. The

numbering of the peptide sequences shown, obtained from published manuscripts, are as follows: cBEN, 8-484 (Pourquie et al, Proc. Natl. Acad. Sci. USA 89:5261 (1992)); neurolin, 1-466 (Lawssing et al, Differentiation 56:21 (1994)); RAGE, which contains three Ig domains, 30-307 (Neeper et al, J. Biol. Chem. 267:14998 (1992)); MUC18, 40-525 (Lehmann et al, Proc. Natl. Acad. Sci. USA 86:9891 (1989)). Fig. 26B. 15  $\mu$ g of total RNA from peripheral blood monocytes and 25  $\mu$ g of total RNA from resting and PHA activated (72h) peripheral blood mononuclear cells, the T cell lymphomas CEM and MOLT4, the erythroleukemia cell line K562, the B cell lymphomas RAMOS, RAJI and DAUDI, the myelomonocytic cell lines HL60 and U937, the large granular lymphoma YT, the human breast carcinoma HBL-100 and COS cells were used to prepare an RNA blot. Random primed <sup>32</sup>P-labeled ALCAM or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA's were used as probes. Fig. 26C. Peripheral blood mononuclear cells were activated *in vitro* with PHA and the ability of T cells to bind to either CD6-Rg or J4-81 was monitored for a period of ten days by two color immunofluorescence and flow cytometry. The mean channel fluorescence vs. day was plotted.

Figure 27. Human chromosomal mapping. Fluorescence *in situ* hybridization (FISH) of cDNA probes to R-banded chromosomes generated signals on both chromatids (indicated by arrows) on chromosome 3 at bands q13.1-q13.2. The region is shown by brackets on a G-banding ideogram.

Figure 28. Binding of ALCAM to CD6. Fig. 28A. Flow cytometry histograms showing the binding of the anti-ALCAM mAb J4-81 and the CD6-Rg fusion protein to COS cell transfectants expressing ALCAM. Fig. 28B. Flow cytometry histograms showing the binding of anti-CD6 mAb G3-6 and ALCAM-Rg to COS cell transfectants expressing CD6. The level of background binding of mAb's or fusion proteins to mock transfected COS cells is shown by shaded histograms.

10 Figure 29. ALCAM encoding sequence and predicted amino acid sequence.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a molecule that represents one member of a binding pair, the other member of that pair being the CD6 molecule present on the surface of mature thymocytes, peripheral T cells and a subset of B cells. The CD6 ligand, as it is referred to herein, occurs in two forms - one form is independent of divalent cations for CD6 binding, the other is divalent cation-dependent. The first of these forms (the cation-independent form) comprises a 105 kDa protein (as determined by SDS-PAGE under reducing conditions) and is termed activated leucocyte-cell adhesion molecule (ALCAM). The second form is divalent cation-dependent, is a distinct molecule separate from ALCAM, and comprises a 90 kDa protein (determined by SDS-PAGE under reducing conditions). The molecular weight of ALCAM based on the predicted amino acid

sequence shown in Figure 29 is about 65 kDa. The amino acid sequence of ALCAM shown in Figure 29 includes 9 potential sites for N-linked glycosylation. Thus, the glycosylated form of the ALCAM CD6 ligand can be expected to have a molecular weight of 100-105 kDa.

ALCAM consists of an N-terminal hydrophobic signal peptide, which is cleaved from the mature protein, followed by an extracellular domain, a hydrophobic transmembrane domain and a cytoplasmic domain. Single domains or combinations of domains can be used to inhibit a variety of interactions. For example, extracellular domains, separately or in combination with each other or other sequences, can be expected to be useful products for inhibiting CD6-ALCAM interactions. The extracellular domain of ALCAM can be divided into 5 immunoglobulin (Ig)-like domains. The two N-terminal domains are of the V-set, the three Ig domains that follow are of the C2-set (Pourquie et al, Proc. Natl. Acad. Sci. USA 89:5261 (1992); Tanaka et al, Neuron 7:535 (1991); Burns et al, Neuron 7:209 (1991)). These Ig-like domains can be used in isolation or in combination with other sequences.

The CD6 ligand (ALCAM) of the invention is present on a variety of tissue and cell types, including fibroblasts, skin epidermal keratinocytes, medullary TEC, gut epithelium, pancreas islet and acinar cells, as well as monocytes and activated T cells, hepatocytes and neurons of the brain cortex. The tissue distribution of the CD6 ligand indicates that the CD6/CD6 ligand system is important for mediation of T cell interactions with monocytes and other activated T cells as well as with

epithelial cells, fibroblasts and specialized cells, such as pancreatic islet cells. The expression of CD6 and CD6 ligand in brain indicates that the CD6/CD6 ligand system is involved in interactions between cells of the nervous system.

The CD6 ligand of the invention can be isolated from natural sources (see above) by immunoprecipitation, for example, with a CD6 fusion protein as described in the Examples that follows. A suitable fusion protein, the CD6-Ig (= CD6-Rg) fusion protein, can be produced by ligating the coding sequence of the extracellular portion of CD6 (Aruffo et al, J. Exp. Med. 174:949 (1991)) to the coding sequence of the Fc portion of human IgG as described by Seed and Aruffo (Proc. Natl. Acad. Sci. USA 84:3365 (1987)). The CD6-Ig fusion protein can be expressed in COS cells by transient transfection as previously described (Seed and Aruffo, Proc. Natl. Acad. Sci. USA 84:3365 (1987)).

The availability of the CD6 ligand protein from cell sources, including those noted above, makes possible the cloning of the CD6 ligand gene(s). Sequencing of all or part of the CD6 ligand protein provides the information necessary for designing nucleic acid probes or primers suitable for use in isolating the CD6 ligand coding sequence(s), for example, from cDNA or genomic DNA libraries. The Unizap XR system from Stratagene can be used to prepare a suitable cDNA library from thymic epithelial cells. Other libraries that can be used include the brain frontal cortex and liver tumor Hep G2 cDNA libraries from Stratagene.

More specifically, the CD6 ligand can be purified by affinity chromatography. Anti-CD6 ligand mab (5-10 mg) (see, for example, Pesando et al, J. Immunol. 137:3689 (1986)) can be used to prepare an affinity column for CD6 ligand by immobilization of anti-CD6 ligand protein on Sepharose (Bower et al, J. Immunol. 151:5891 (1993)) or CNBr-activated Sepharose 4B (Pharmacia), as described by Patel et al (Virology 149:174 (1986)). CD6 ligand from protein lysates of, for example, breast carcinoma cells (eg HBL-100), thymic epithelial cell or B lymphoma cell line lysates, can be affinity purified using the anti-CD6 ligand Sepharose column. CD6 ligand can be further purified over a C<sub>18</sub> reverse phase HPLC column (Vydac) using acetonitrile/H<sub>2</sub>O gradients containing trifluoroacetic acid with UV detection at 240nm. (Example IV includes a description of the J4-81 immunopurification of CD6 ligand from breast carcinoma cells.)

Once purity has been established, the N-terminal amino acid sequence of intact CD6 ligand, and peptides generated with V8-protease, trypsin or other proteases, can be determined. From the amino acid sequence, degenerate oligonucleotide primers can be designed that recognize cDNA for CD6 ligand, to PCR amplify CD6 ligand cDNA. To guard against errors in the PCR amplification, <sup>32</sup>P-labelled PCR amplified CD6 ligand cDNA can be used as a probe to detect CD6 ligand cDNAs from an appropriate cDNA library. The nucleotide sequence of CD6 ligand cDNA can be determined using standard methodologies. (See generally, Sambrook, Fritsch and

Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press (1989).)

Example IV includes a description of the analysis of purified ALCAM and CNBr fragments thereof in a pulsed-liquid protein sequencer. The amino acid sequences from intact protein and internal fragments are homologous with the chicken neuronal adhesion molecule BEN/SC-1/DM-GRASP. Example IV describes the use of DNA fragments corresponding to chicken BEN to isolate-CD6 ligand cDNA. The ALCAM cDNA sequence and predicted amino acid sequence are shown in Fig. 29.

Once cloned, the CD6 ligand encoding sequence can be introduced into a host cell, for example, in an expression construct (eg, wherein the vector is a viral or plasmid vector) in operable linkage with a promoter. The construct can be used to transform a procaryotic or eucaryotic host cell. Eucaryotic hosts are preferred since the CD6 ligand is a glycosylated protein. Mammalian cell systems are particularly advantageous (eg, the COS M6 system). The CD6 ligand can be produced in soluble form by culturing the host cells transfected with a gene encoding CD6 ligand protein (eg, an Ig fusion protein) under conditions such that the encoding sequence is transcribed and the transcript is translated into protein.

The present invention relates to the CD6 ligand in its entirety and also to portions thereof suitable, for example, for use as antigens that can be used in standard immunization protocols to generate antibodies (monoclonal or polyclonal) (or binding fragments thereof) specific for the CD6 ligand (the ligand and the

portions can be synthesized chemically or recombinantly). Such portions represent at least 5 consecutive amino acids of the CD6 ligand (eg of the ALCAM sequence shown in Figure 29), preferably, at least 10 or 12 amino acids, more preferably, at least 25 consecutive amino acids, most preferably at least 40, 45 or 50 amino acids. Larger portions can also be used, for example, portions of at least 100, 250 or 500 amino acids. The invention includes portions of the CD6 ligand corresponding to the signal sequence, or the extracellular, transmembrane or cytoplasmic domain, alone or in combination with other CD6 ligand domain sequences or non-CD6 ligand sequences.

The invention also relates to a CD6 ligand encoding sequence (DNA or RNA) in its entirety and to portions thereof suitable for use, for example, as probes or primers (hybridization conditions described as standard by Strategene and/or Gibco (eg for PCR) can be used). Such portions represent at least 15, 20, 30, 75 or 150 nucleotides of the CD6 ligand (for example, of the sequence given in Figure 29). Larger portions can also be used, for example, portions of at least 300, 750 or 1500 nucleotides. The invention includes portions of the CD6 ligand encoding sequence corresponding to the signal sequence or the extracellular, transmembrane, or cytoplasmic domain. Such portions can be present in various combinations and in combination with non-CD6 ligand encoding sequences (eg, ALCAM-Rg).

Ligation of CD6 on T cells by anti-CD6 mab provides a potent co-mitogenic signal for unseparated T cells (Gangemi et al, J. Immunol. 143:2439 (1989); Morimoto et



al, J. Immunol. 140:2165 (1988); Wee et al, J. Exp. Med. 177:219 (1993)), directly activates separated CD4+ T cell clones (Swack et al, J. Biol Chem. 266:7137 (1991)), and directly activates TCR $\gamma\delta$  but not TCR $\alpha\beta$  T cells (Pawlec et al, Hum. Immunol. 31:165 (1991)). Thus, CD6 is a molecule of mature T cells that is intimately involved in TCR mediated T cell triggering; ligation of the TCR complex leads to CD6 phosphorylation (Wee et al, J. Exp. Med. 177:219 (1993)). Methods of inhibiting CD6/CD6 ligand interactions in vivo provides a potent immunotherapeutic strategy.

Soluble CD6 ligand (isolated from natural sources or produced recombinantly) can be used, for example, to inhibit CD6-mediated T cell activation that is dependent on T cell CD6-CD6 ligand + accessory cell contact. A portion of the CD6 ligand can also be used; for example, a domain such as the extracellular domain can be used alone or in combination with other domains or other sequences. Such an immunotherapeutic regimen is useful in treating diseases caused by activated T cells such as multiple sclerosis, inflammatory uveitis, including Cogan's syndrome, rheumatoid arthritis, T cell mediated vasculitis syndromes, such as Wegener's granulomatosis and temporal arteritis, and organ allograft rejection. Indeed, it has been shown that CD6 mab depletion of bone marrow (BM) used for allogeneic BM transplant prevents graft versus host disease (Soiffer et al, J. Clin. Oncol. 10:1191 (1992)).

In addition to soluble CD6 ligand, soluble CD6, as well as mimetopes (mimics) of CD6 and CD6 ligand (prepared, for example, as described by Szostak (TIBS

17:89 (1992)) or Tsai et al (J. Immunol. 150:1137 (1993)), for example, from random RNA, DNA or peptide libraries), can be used as immunotherapeutic agents in regimens involving inhibiting CD6/CD6 ligand interaction. Anti-CD6 and anti-CD6 ligand antibodies (preferably monoclonal antibodies) can also be used in such regimens. These immunotherapeutic agents can be formulated with, for example, a pharmaceutically acceptable carrier, diluent, etc., as a pharmaceutical composition. The concentration of active agent in the composition and the amount of active agent administered will vary with the agent, the patient and the effect sought. Optimum doses can be readily determined.

The demonstration that CD6-CD6 ligand interactions mediate the adhesion of CD6 expressing cells to TE cells indicates that CD6-CD6 ligand binding plays a role in regulating T cell maturation. Other molecules including CD2/LFA-3, ICAM-1/LFA-1, VLA-3, -4, -6, and fibronectin can serve as adhesion molecules in thymocyte-stromal cell binding (Singer et al, J. Immunol. 144:2931-2939 (1990), Giunta et al, J. Exp. Med. 173:1537-1548 (1991), Utsumi et al, Proc. Natl. Acad. Sci. USA 88:5685-5689 (1991)). The identification of a CD6 ligand permits determination of the relative contribution of CD6 in thymocyte-TE cell binding and T cell maturation. CD6-ligand may also be involved in B cell development, since studies in the chicken showed that ALCAM (BEN) is expressed on endothelial cells in the bursa of Fabricius (Pourquie et al, Development 109:743-752 (1990)), which is an organ in birds where B cells develop. Presently, there is no information on the ability of CD6 ligand to

transduce intracellular signals. The finding that the anti-CD6 mAB T12 can activate T cells in the presence of accessory cells and in the apparent absence of crosslinking FcR's, indicates that activation of T cells by signaling through CD6 is important in autoimmune reactivity (Gangemi et al, J. Immunol. 143:2439-2447 (1989)). In support of this, anti-CD6 mAB's can enhance or inhibit the autologous MLR (Gangemi et al, J. Immunol. 143:2439-2447 (1989), Bott et al, Int. Immunol. 7:783-792 (1994)) (Singer, N.G. et al, J. Immunol. (1994)). Since CD6 ligand is expressed by activated T cells and monocytes as well as by a number of T and B cell lines, CD6-CD6 ligand interactions would appear to play a role in functional interactions between CD6+T and B cells and activated leukocytes and CD6-CD6 ligand binding would appear to mediate adhesive interactions among activated leukocytes. Thus, while ALCAM may not affect the initiation of an inflammatory response, it may inhibit maintenance of a chronic inflammatory response, such as rheumatoid arthritis.

ALCAM (BEN) in the chicken is expressed predominantly during early embryonic development and in the brain (Pourquie et al, Development 109:743-752 (1990)). ALCAM functions as a homophilic adhesion molecule and supports neurite outgrowth (Tanaka et al, Neuron 7:535-545 (1991), Burns et al, Neuron 7:209-220 (1991)). Expression of human ALCAM by neurons in the brain has been reported (Patel et al, "Identification and characterization of an 100 kDa ligand for CD6 on human thymic epithelial cells", J. Exp. Med. (1994)). Interactions between the immune and nervous system are

important in the pathology of certain chronic neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and amyotrophic lateral sclerosis (Appel et al, Advances in Neurology 56:405-412 (1991),  
5. McGeer et al, Can. J. Neurol. Sci. 18:376-379 (1991), Rowland, L.P., Advances in Neurology 56:3-23 (1991)). The finding that CD6 and CD6 ligand are both expressed by cells in these systems indicate that this  
10 receptor/ligand pair functions in cellular interactions between the immune and nervous systems.

For example, since inflammation requires amplification of immune responses, blocking CD6 binding can be expected to prevent immune response amplification and inflammation. Further, inhibition of leukocyte  
15 interactions with cells of the brain can be used to treat chronic neurodegenerative diseases. Mutations in ALCAM can be expected to lead to disorders of the nervous and/or immune systems. When such disorders are identified, the CD6 ligand nucleotide sequence or RFLP's  
20 (restriction fragment length polymorphisms) identified for the CD6 ligand can be used as markers for candidate gene searches. For example, since ALCAM is expressed on motor neuron's and immune cells, candidate diseases to screen for ALCAM mutations are sporadic cases of  
25 amyotrophic lateral sclerosis and multiple sclerosis.

In addition to the above, it will be appreciated from a reading of this disclosure that soluble ALCAM, for example, can be used to induce regeneration of damaged or severed nerves. Induction can be effected by  
30 administering soluble ALCAM at the site of nerve damage, for example, by injection.

The CD6 ligand gene is a potential candidate for mutations in Mendelian disorders affecting the nervous system and immune system. No disease suggestive for further testing has yet been mapped to that region of human chromosome 3 to which the CD6 ligand gene has been assigned. The polymorphisms identified in the CD6 ligand cDNA can be used for candidate gene exclusion studies. (Polymorphisms noted may result from the fact that cDNAs were obtained from a PHA activated cDNA library and HL60 cells. Different donor sources often show polymorphisms in genes.)

Certain aspects of the invention are described in greater detail in the non-limiting Examples that follows.

15

EXAMPLE IExperimental details:

*Cells and culture conditions.* TEC were cultured by the explant technique in enriched media as previously described (Singer et al, Human Immunol. 13:161 (1985)). Human thymus tissue was obtained from the Department of Pathology, Duke University Medical Center, as discarded tissue from children undergoing corrective cardiovascular surgery. Contaminating thymic fibroblasts were removed by complement mediated lysis with mAb 1B10 which binds to a cell surface antigen on human fibroblasts (Singer et al, J. Invest. Dermatol. 92:166 (1989)) followed by treatment with 0.02% EDTA in PBS. 3T3 fibroblast feeder layers were removed by

treatment with 0.02% EDTA in PBS prior to detachment of TEC from culture dishes with 0.05% trypsin in PBS containing 0.02% EDTA. Cells were washed 3 times prior to analysis. TEC were activated with 500 U/ml IFN- $\gamma$  in DMEM containing 5% FCS, 1 mM sodium pyruvate (Gibco) 0.025  $\mu$ g/ml amphotericin B (Gibco), 100 U/ml penicillin and 1000  $\mu$ g/ml streptomycin for 48-72h at 37°C.

Thymocytes were obtained by teasing from thymus tissue, purified by centrifugation through ficoll-hypaque and washed with RPMI 1640. Thymocytes were used immediately or frozen in media containing 20% FCS, 7.5% DMSO and 10  $\mu$ g/ml gentamicin (Scherring) in RPMI 1640. Frozen thymocytes were thawed by incubation in media containing 30% FCS, 10  $\mu$ g/ml deoxyribonuclease I (Sigma), 10  $\mu$ g/ml gentamicin and 20 U/ml heparin (Upjohn) in RPMI 1640 as described (Denning et al, J. Immunol. 138:680 (1987)). Viable thymocytes were purified by centrifugation through ficoll-hypaque.

COS-M6 cells (ATCC) were grown in DMEM containing 10% FCS, 1 mM sodium pyruvate (Gibco), 0.025  $\mu$ g/ml amphotericin B (Gibco), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin.

Human epidermal keratinocytes (EK) were cultured from neonatal foreskins. Foreskins were incubated at 4°C overnight in 2.5 mg/ml trypsin type III (Sigma) in HBSS (GIBCO), the epidermis was removed, teased into a single cell suspension, seeded onto mitomycin C treated 3T3 fibroblast feeder layers and cultured as described (Hashimoto et al, J. Exp. Med. 157:259 (1983)).

*Detection of cell surface antigens:* Unfixed cultured cells were suspended in either PBS, TBS (0.9 M NaCl, 50 mM Tris-HCl, pH 7.3) or DMEM containing 2% BSA and 0.1% NaN<sub>3</sub>. Cells were incubated with CD6-Rg, CD5-Rg or ELAM-Rg (from Sandro Aruffo, Bristol-Myers Squibb), recombinant fusion proteins (100 µg/ml) for 30 min at 4°C and washed with PBS containing 2% BSA and 0.1% NaN<sub>3</sub>. FITC conjugated goat anti-human IgG<sub>1</sub> was used as a secondary reagent. Cells were analyzed on a FACStar Plus flow cytometer (Becton-Dickinson, Inc., Mountain View, California) and data was processed using the software program PC-Lysys (Becton-Dickinson).

*TEC-thymocyte rosette binding assay:* TEC-thymocyte rosette binding assays were performed as described (Singer et al, Proc. Natl. Acad. Sci. USA 83:6588 (1986)). Briefly, 10<sup>6</sup> thymocytes were mixed with 2 x 10<sup>5</sup> TEC in PBS or DME containing 2% BSA and 0.1% NaN<sub>3</sub>, centrifuged for 3 min at 250g, and incubated for 60 min at 4°C. Cells were gently resuspended and counted under light microscopy. TEC which bound 3 or more thymocytes were scored as positive. The thymocytes used in these experiments were either freshly isolated, thawed from liquid nitrogen, or separated into subpopulations. Thymocytes were separated into CD1<sup>+</sup> (immature, CD6<sup>low</sup>) or CD1<sup>-</sup> (mature, CD6<sup>hi</sup>) subpopulations by indirect immunofluorescent staining with Na1/34 (anti-CD1; from Andrew McMichael, Oxford, England) and goat anti-mouse IgG (KPL) followed by fluorescence activated cell sorting using a FACStar Plus. All thymocyte subpopulations were >95% pure on

reanalysis of sorted cells. TEC-thymocyte binding was inhibited by preincubation of either thymocytes or TEC with antibodies or fusion proteins prior to binding.

5. Antibodies used were: P3X63 (control mab) (ATCC), 35.1 (anti-CD2; from John Hansen, Seattle, Washington (Martin et al, J. Immunol. 131:180 (1983)) and T12 (anti-CD6; from Ellis Reinherz, Boston, Massachusetts (Reinherz et al, Cell 30:735 (1982))).

- 10 *COS cell transfection and binding studies:* Stable lines of CD6-expressing COS cells were made by co-transfection of plasmids containing the CD6 gene under the control of the CMV promoter (A. Aruffo, Seattle, Washington Aruffo et al, J. Exp. Med. 174:949 (1991)) and the pSVneo plasmid containing the bacterial neomycin resistance gene driven by an SV40 promoter as described
- 15 by Liao et al (J. Immunol. December 1, 1993). Cells expressing the neomycin resistance gene were selected by incubation in DME/10%FCS containing 60µg/ml G418 (GIBCO). Cells expressing CD6 were identified by
- 20 indirect immunofluorescence using mab T12. CD6 positive and negative cells were cloned by single cell sorting using a Becton-Dickinson FACStar<sup>Plus</sup> flow cytometer.

- CD6-expressing COS cells (COS-CD6D) and control COS cells (COS-Neo) were used in binding studies, with TE
- 25 cells similar to those outlined above. To differentiate between COS cells and TE cells, TE cells were metabolically labelled with 1 µg/ml calcein AM (Molecular Probes, Eugene, Oregon) for 15 min at 37°C in PBS prior to harvest. Calcein AM-labelled cells are



fluorescent and can be easily differentiated from other cells by fluorescence microscopy.

*Construction of the CD6-Rg (CD6-Ig) chimeric gene and preparation of the fusion protein:* The CD6 immunoglobulin fusion gene was constructed by digesting a plasmid containing the cDNA encoding the full-length CD6 (Aruffo et al, J. Exp. Med. 174:949 (1991)) with the restriction enzyme Esp1. The Esp1 digested plasmid was then flushed with DNA polymerase and the appropriate Bam HI linkers were added to it. The plasmid was then digested with Bam HI and Nde 1. The fragment that contained the extracellular domain of CD6 was then isolated and subcloned into a vector containing a cDNA fragment encoding the constant domain of a human IgG1 protein (Aruffo et al, Proc. Natl. Acad. Sci. USA 89:2292 (1992)). The constructions of CD5-Ig and CTLA4-Ig have been described elsewhere (Linsley et al, Science 257:792 (1992) and J. Exp. Med. 174:561 (1991)). 500 µg of DNA of the appropriate gene was transfected into COS cells by the DEAE-dextran chloroquine method. 18-24 hrs later, the 10% FBS Dulbecco's modified Eagle's medium was replaced by serum-free media after a rinse wash with PBS. The cell culture was maintained 7-10 days before it was harvested. Briefly, the supernatant containing the fusion protein was centrifuged at 2500 rpm for 20 min and filtered through a 0.45 µm Millipore filter. The supernatant was passed over a 2-ml packed Protein A column at a flowrate of 1.00 ml/min. The bound fusion protein was eluted with pH 3.5, 0.1 M citric acid and was immediately neutralized with pH 9.0, 1.0 M Tris.

The eluted protein was then dialyzed overnight in PBS and protein determination was done using the Bradford method.

5                    *Screening of mabs for inhibition of CD6-Ig binding*  
to TE cells: Mabs from the 5th International Workshop  
on Human Leukocyte Differentiation Antigens and a panel  
of anti-integrin mabs (see description of Figure 4) were  
screened for reactivity to the surface of TE cells as  
outlined above, using fluorescein-conjugated goat anti-  
10 mouse IgG (KPL) as a secondary reagent. Of the 154 mabs  
that reacted to TE cells, 126 were used in this assay.  
TE cells (100k) were incubated with ascites or purified  
mab at 1:100 for 15 min at 4°C. Either CD5-Ig or CD6-Ig  
(5 µg) was added to this mixture and allowed to react  
15 for 2 hr at 4°C. After washing with PBS-containing 2%  
BSA, CD5-Ig and CD6-Ig were labelled with a fluorescein  
conjugated goat antiserum specific for the Fc protein of  
human IgG (Sigma). This reagent did not cross react  
with most murine mabs. To account for any cross-  
20 reactivity that may have occurred, binding was  
determined to be the difference in fluorescence (ΔFL)  
between samples containing CD6-Ig and CD5-Ig. The ΔFL  
of samples pre-incubated with control mab P3 was  
considered to represent 100% binding.

25                    *Immunoprecipitation and protein labelling*  
conditions: 120 µCi/ml glucosamine (NEN) was used in  
the labelling experiment. The cells were cultured in  
glucose-free media plus 10% dialyzed fetal calf serum  
for 48 hrs upon addition of the radiolabelled

glucosamine. The cells were then lifted with EDTA, washed in HBSS and lysed in 20 mM Tris, pH 7.5 containing leupeptin, PMSF and pepstain (1  $\mu$ g/ml), 0.05% sodium cholate, 2% NP-40. The lysate was pre-cleared with 100  $\mu$ g/ml human IgG1 followed by 50  $\mu$ l of Protein A beads. Pre-cleared lysates were then incubated with 50  $\mu$ g/ml CD6-Rg or CTLA4-Ig or 2  $\mu$ g/ml anti-EGF receptor antibody and Protein A Sepharose. 2 mM Ca++ and Mg++ were added to the lysates (this is the concentration of divalent cations contained in the HBSS binding buffer). The immunoprecipitates were washed 3x in lysis buffer, 2x in PBS, boiled in reducing SDS-PAGE loading buffer and loaded unto a 8-10.5% SDS-PAGE gel. The gels were fixed in 40% isopropanol, enhanced in m AMiAmplify<sup>R</sup> reagent (Amersham), dried and autoradiographed.

### Results:

*Anti-CD6 mabs and CD6-Ig fusion proteins inhibit TE-thymocyte binding:* Using a suspension TE-thymocyte binding assay (Table 1), it was found that the CD6 mab T12 inhibited TE-thymocyte binding by 49 $\pm$ 9% (N=5). Similarly, recombinant CD6-Ig fusion protein inhibited TE-thymocyte binding by 35 $\pm$ 9% (N=5). This suggested that human TEC express a ligand for CD6.

Table 1

Effect of monoclonal antibodies and fusion protein  
on thymocyte-thymic epithelial cell binding

mab/fusion protein	% Binding <sup>a</sup>	% Inhibition <sup>b</sup>
P3	32.8 ± 5.2	0
35.1 (anti-CD2)	8.2 ± 2.4	76.0 ± 5.4 p<0.003
T12 (anti-CD6)	17.2 ± 3.8	48.7 ± 9.2 p<0.015
ELAM-IG	29.7 ± 3.6	0
CD6-IG	19.3 ± 3.2	34.5 ± 9.3 p<0.05

<sup>a</sup>Binding was determined to be the percentage of TE cells rosettes ( $\geq 3$  bound thymocytes). Shown is the mean and standard error of 5 separate experiments.

<sup>b</sup>% Inhibition =  $100 \times (\text{Binding}_{\text{control}} - \text{Binding}_{\text{exp}}) / \text{Binding}_{\text{control}}$ . The controls were P3 for mabs, and ELAM-Ig for CD6-Ig.

P values represent 2 tailed Student's t-test comparing binding in the presence of mabs or CD6-Ig to control binding.

Human TE cells express a ligand for CD6: To confirm that human TEC express a ligand for CD6, TEC were incubated with recombinant CD6-Ig fusion proteins and assayed for CD6-Ig binding to TEC by indirect immunofluorescence followed by flow cytometry. Neither

5 of the negative controls (CD5-Ig and ELAM-Ig) bound to

the surface of TEC while CD6-Ig did bind to TE cells (Table 2), indicating that there is a ligand for CD6 expressed on the surface of human TE cells. CD6-Ig binding to the CD6 ligand on TE cells was enhanced by divalent cations ( $\text{Ca}^{++}$  and  $\text{Mn}^{++}$ ) and partially inhibited by EDTA (Figures 1 and 2). The data suggest that there are at least two components to the binding of CD6-Ig to TEC - a component (or ligand) that is dependent upon divalent cations, and a component (or ligand) that is divalent cation-independent.

Table 2

Reactivity of fusion proteins on  
human thymic epithelial cells<sup>a</sup>

fusion protein	MFC <sup>b</sup>
IgG <sub>1</sub>	72.9
ELAM-Ig	70.5
CD5-Ig	72.1
CD6-Ig	164.9

<sup>a</sup>Cultured TE cells were incubated with 2000  $\mu\text{g}/\text{ml}$  fusion proteins. Fusion proteins were detected by indirect immunofluorescence followed by flow cytometry.

<sup>b</sup>Linear Mean Fluorescence Channel. Data are representative of 3 experiments.

CD6 is an adhesion molecule: The fact that T12 and CD6-Ig both partially inhibit the binding of thymocytes to TEC strongly suggested that CD6 was an adhesion molecule. The TE rosette studies, however, did not rule out the possibility that T12 and CD6-Ig inhibited TE-thymocyte binding because of steric hinderance. To determine if CD6 was indeed an adhesion molecule, a COS cell line (COS-CD6D) was made that expressed high levels of transfected CD6. COS cells without CD6 (CS-Neo) did not bind to TEC, whereas COS-CD6D cells did bind TEC (Figure 3). Furthermore, this COS-CD6D:TE cell binding was CD6-dependent as binding was inhibited by mab to CD6 (T12).

Mab J4-81 reacts with a ligand for CD6: To identify the CD6 ligand, a large panel of anti-integrin mabs and a blind panel of 479 mabs from the Vth International Workshop on Human Leukocyte Differentiation Antigens were screened for reactivity to TE cells. Of the 154 mabs that reacted with TEC, 126 mabs were used in assays to inhibit the binding of CD6-Ig to TEC. As shown in Figure 4, only Workshop mab S252 (J4-81) was able to inhibit the binding of CD6-Ig to TEC. Mab J4-81, which was raised against B cell surface antigens, recognizes a 105 kDa protein on B cells (Pesando et al, J. Immunol. 137:3689 (1986)) and reacts strongly with the surface on all cultured TEC (Figure 5) and with medullary TEC in frozen sections of human thymus (Figure 6).

Immunoprecipitation of 105 kDa, 90 kDa and 35 kDa molecules from TEC with CD6-Ig: In the absence of EDTA, CD6-Ig fusion protein immunoprecipitated protein species with molecular weights of 105, 90 and 35 kDa (Figure 7, lane 1) (note approximately 150 kDa band). In the presence of EDTA, CD6-Ig immunoprecipitated only the 105 and 35 kDa species (Figure 7, lane 2) (note approximately 150 kDa band). In lane 3, in the absence of EDTA, the control fusion protein, CTLA4-Ig, did not immunoprecipitate the 105, 90 or 35 kDa protein species.

Mab J4-81 inhibits the binding of TE'cells to COS-CD6D cells: A third approach has been used to define that the 105/35 kDa protein molecules detected by mab J4-81 is a ligand for CD6. As shown above, COS-CD6D:TEC binding is CD6-specific. To further demonstrate that CD6 and the 105/35 kDa molecules recognized by J4-81 form an adhesion molecule pair, COS-CD6D:TEC binding with mab J4-81 was inhibited (Table 3).

Table 3

Effect of monoclonal antibodies COS-CD6D:TEC binding

mab	% Binding <sup>a</sup>	% Inhibition <sup>b</sup>
P3	25.0 ± 1.8	0
T12 (anti-CD6)	4.5 ± 0.7	82.7 ± 2.1 p<0.0007
S252 (J4-81)	8.7 ± 0.7	65.3 ± 1.8 p<0.0008

<sup>a</sup>Binding was determined to be the percentage of COS cells rosettes ( $\geq 3$  bound TEC). Shown is the mean and standard error of 3 separate experiments.

<sup>b</sup>% Inhibition =  $100 (\text{Binding}_{\text{P3}} - \text{Binding}_{\text{exp}}) / \text{Binding}_{\text{P3}}$ .

P values represent 2 tailed Student's t-test comparing inhibition in the presence of T12 or J4-81 inhibition in the presence of P3.

Reactivity of J4-81 mab with human tissue in indirect IF assay: Mab J4-81 reacted with TEC, epidermal keratinocytes, fibroblasts, acinar cells and islets cells of pancreas, gut epithelium, monocytes (10%) and activated PB T cells (21%). B cells are also positive for J4-81 (Pesando et al, J. Immunol. 137:3689 (1986)). Taken together, these data demonstrate that the CD6-CD6 ligand system is involved in TEC-thymocyte interactions, that CD6 is an adhesion molecule and that 105/35 kDa proteins detected by mab J4-81 comprise a ligand for CD6. Moreover, that the CD6-divalent cation-independent



ligand defined by mab J4-81 is expressed on a wide variety of immune and epithelial cell types suggests that the CD6/CD6 ligand system is important in immune cell interactions with other immune cells and with a wide variety of epithelial microenvironments.

## EXAMPLE II

### Experimental details:

*Cell lines, fusion protein, and antibodies.* The colon carcinoma-derived cell line H3719 and the melanoma-derived cell line H3606 were from Drs. K.E. and I. Hellstrom (Bristol-Myers Squibb, Seattle, WA). The human breast epithelial cell line HBL-100, the EBV-transformed human B cell line LCL, the human lung fibroblast IMR90, the adult T cell leukemia Jurkat, the cutaneous T cell lymphoma HUT78, the human peripheral blood acute leukocytic leukemia derived cell line HPB-All, the human T cell lymphoma H9, the human Burkitt lymphoma Raji, the fibroblast cell line GM0833, the B cell lymphoblastoma Peer, the T cell leukemia JM, and the B cell lymphoblastoma LTR228 were used and obtained primarily from the American Type Culture Collection (Rockville, MD). The CD5-Rg (Aruffo et al, Proc. Natl. Acad. Sci. USA 89:10242 (1992)), ELAM1-Rg (Walz et al, Science 250:1132 (1990)), and CTLA4-Ig (Linsley et al, J. Exp. Med. 174:561 (1991)) have been previously described. The anti-CD6 mAb G3-6 (IgG) was from Dr. J. Ledbetter (Bristol-Myers Squib, Seattle, WA) (Ledbetter et al, Proc. Natl. Acad. Sci. USA 84:1384 (1987)); the

anti-CD6 mAb MBG6 (IgM) was from Dr. A. McMichael (Oxford University, Oxford, U.K.) (Bastin et al, J. Clin. Exp. Immunol. 46:597 (1981)); the anti-CD6 mAb 2H1 was from Dr. C. Morimoto (Dana-Farber Cancer Institute, Boston, MA) (Morimoto et al, J. Immunol. 140:2165 (1988)); the anti-CD6 mAbT12 has been previously described (Reinherz et al, Cell 30:735 (1982)). The DNA restriction enzymes and the DNA linkers were obtained from New England Biolabs (Beverly, MA); RPMI, HBSS, FBS, and prestained molecular weight markers were obtained from GIBCO-BRL (Gaithersburg, MD). Human epidermal keratinocytes (EK) were cultured from neonatal foreskins. Foreskins were incubated at 4°C overnight in 2.5 mg/ml trypsin type III (Sigma, St. Louis, MO) in HBSS (GIBCO-BRL), the epidermis was removed, teased into single-cell suspension, seeded onto mitomycin C-treated 3T3 fibroblast feeder layers, and cultured as described (Hashimoto et al, J. Exp. Med. 145:259 (1983)).

*Preparation of CD6-Rg.* The plasmid encoding the CD6-Rg containing a full-length cDNA clone encoding CD6 (Aruffo et al, J. Exp. Med. 174:949 (1991)) was digested with the restriction enzyme *EspI*, treated with the Klenow fragment of DNA polymerase I, and ligated to *BamHI* linkers (New England Biolabs, Beverly, MA). The vector was then digested with the restriction enzymes *NdeI* and *BamHI*. The *NdeI/BamHI* DNA fragment containing the extracellular domain of CD6 was subcloned into a plasmid containing a cDNA fragment encoding the constant domains (hinge, CH2, and CH3) of human IgG<sub>1</sub>. The CD6-Rg protein was prepared by transient expression in COS

cells as previously described (Aruffo et al, Cell 61(7):1303 (1990)) and purified from the supernatant of COS cell transfectants by absorption to, and elution from, a protein A column (Repligen, Cambridge, MA).

5            *CD-6 Rg cell binding studies.* Typically,  $5 \times 10^7$  cells/ml in HBSS/2% FBS/20 mM Hepes (wash/stain buffer) were incubated with 50  $\mu$ g/ml of fusion protein. Adherent cells were detached from dishes with 0.5 mM EDTA in PBS (PBS/EDTA) and washed once, while  
10 nonadherent cells were washed once before incubation with the fusion protein for 1 hr on ice. The cells were subsequently washed three times and incubated with FITC-goat anti-human IgG (10  $\mu$ g/ml, Tago, Burlingame, CA) for 1 hr on ice. The cells were washed three times, fixed  
15 with 1% paraformaldehyde in PBS, and analyzed by flow cytometry (Epics V, Coulter, Hialeah, FL).

          This staining procedure was used to examine if the binding of CD6-Rg to HBL-100 cells was saturable. In those experiments the CD6-Rg and control fusion protein  
20 were used at the following concentrations: 10, 20, 30, 40, 50, 100, and 200  $\mu$ g/ml. To investigate the sensitivity of CD6-Rg ligand(s) to trypsin, HBL-100 cells ( $5 \times 10^7$ ) were treated with 0.05% trypsin/0.5 mM EDTA for 30 min at 37°C, washed three times with  
25 wash/stain buffer, incubated with CD6-Rg and analyzed by flow cytometry as described above. To examine the divalent cation requirement for CD6-Rg binding, CD6-Rg was incubated with HBL-100 cells in the presence of 15 mM EDTA, or, alternatively, the EDTA was added to  
30 HBL-100 cells which had been previously incubated with

CD6-Rg and washed. For anti-CD6 blocking experiments CD6-Rg (50  $\mu\text{g/ml}$ ) was incubated with a 1:50, 1:100, and 1:200 dilution of the 1 mg/ml anti-CD6 mAb G3-6 (IgG) or ascites containing the MBG6 mAb (IgM, from A.J. McMichael) or an isotype matched (IgM) antibody. To examine the cytokine-induced modulation of CD6-Rg binding to HBL-100 cells, cells were incubated with IL-1 $\beta$ , TNF- $\alpha$  (Genzyme, Boston, MA), and IFN- $\gamma$  (Upstate Biotechnology Inc., Lake Placid, NY) (10 ng/ml) individually, in pairwise combinations, or all together, for 48 hr at 37°C, 5% CO<sub>2</sub>, prior to CD6-Rg binding studies.

*Immunoprecipitation studies.* For immunoprecipitation studies, cells were incubated with 120  $\mu\text{Ci/ml}$  of [6-<sup>3</sup>H]glucosamine (NEN Dupont, Boston, MA) in glucose-free RPMI containing 10% normal RPMI and 10% dialyzed FBS for 48 hr at 37°C and 5% CO<sub>2</sub>. The cells were lifted from the culture dish with PBS/EDTA, washed in HBSS, and lysed in 20 mM Tris, pH 7.5, containing leupeptin, PMSF, and pepstatin (1  $\mu\text{g/ml}$ , Boehringer Mannheim, Indianapolis, IN), 0.05% sodium cholate, 2% NP-40 (lysis buffer). The lysates were spun to remove nuclei and precleared by incubating two times with 50  $\mu\text{g/ml}$  of human IgG1 (Sigma) and 50  $\mu\text{l}$  of protein A-Sepharose slurry (Repligen) for 30 min at 4°C. Cell lysates were then incubated with 50  $\mu\text{g/ml}$  of CD6-Rg or CTLA4-Ig or 2  $\mu\text{g/ml}$  of an anti-EGF receptor antibody (Oncogen Science, Uniondale, NY) in the presence of either 2 mM Ca<sup>2+</sup> and Mg<sup>2+</sup> or 15 mM EDTA with 50  $\mu\text{l}$  of protein A-Sepharose (Repligen), for 2-4 hr at 4°C. The

protein A-Sepharose beads were then washed three times with lysis buffer. The immunoprecipitated proteins were analyzed by SDS-PAGE (8-10.5% gradient gel) followed by autoradiography.

5            *Immunohistology.* Murine (Balb/c) lymphoid and nonlymphoid tissues were removed and frozen in liquid nitrogen. Six-micrometer cryostat tissue sections were prepared, mounted on glass slides, and fixed with ice-cold acetone. The fixed sections were stained using  
10        50 µg/ml of CD6-Rg or human IgG1 (Sigma) in a solution of PBS containing 1 mM Ca<sup>2+</sup> and Mg<sup>2+</sup>, 10% BSA, and 10% normal goat serum (NGS) (staining buffer) for 1 hr at room temperature. The slides were washed three times in staining buffer and incubated with a 10 µg/ml of  
15        fluorescein-conjugated, affinity-purified goat anti-human IgG antibody (Tago) for 1 hr at room temperature. After washing three times with staining buffer, the slides were examined by fluorescence microscopy.

#### Results:

20            *CD6 receptor globulin, CD6-Rg.* The CD6-Rg fusion gene was constructed by fusing a cDNA fragment encoding the 400-amino acid extracellular domain of CD6 (Aruffo et al, J. Exp. Med. 174:949 (1991)), including its amino acid terminal signal sequence, onto a cDNA fragment  
25        encoding the hinge (H), CH2, and CH3 domains of human IgG, and subcloned into the mammalian expression vector CDM7B (Aruffo et al, Cell 61(7):1303 (1990)) as described above. The cDNA fragment encoding the IgG

constant domain contains three point mutations. These amino acid substitutions result in the impaired binding of IgG Fc domains to FcRI, II, and III.

5 The CD6-Rg protein used in this study was obtained by transient expression in COS cells and purified by absorption to, and elution from, a protein A column (Aruffo et al, Cell 61(7):1303 (1990)). CD6-Rg is expressed as a covlanet homodimer and is recognized by all anti-CD6 mAb tested in an ELISA assay (T12, 2H1, MBG6, and G3-6). The previously described CD5-Rg (Aruffo et al, Proc. Natl. Acad. Sci. USA 89:10242 (1992)), ELAM1-Rg (Walz et al, Science 250:1132 (1990)), and CTLA4-Ig (Linsley et al, J. Exp. Med. 174:561 (1991)) fusion proteins and/or human IgG1 were used as isotype matched controls in all the binding and immunoprecipitation studies.

*CD6-Rg binding to human and murine cells.* The binding of CD6-Rg to a number of human and murine cell lines was examined by indirect immunofluorescence using flow cytometry. CD6-Rg bound to a subset of the cell lines examined (Fig. 8). Among the cell lines which showed the brightest fluorescence intensity following CD6-Rg staining were the human breast epithelial-derived cell line HBL-100, the human colon carcinoma-derived cell line H3719, the melanoma-derived cell line H3606, the EBV-transformed human B cell line LCL, and the human fibroblast cell lines GM0833 and IMR90. Among the cell lines exhibiting intermediate fluorescence intensity following CD6-Rg binding were the lymphoid cell lines Jurkat, Peer, and HUT78. A number of other lymphoid

cell lines including HPBALL, JM, H9, LTR228, and Raji exhibited no binding to CD6-Rg. None of these cell lines exhibited significant binding to the control fusion protein CD5-Rg. The binding to HBL-100 cell line was further characterized.

Binding of increasing concentrations of CD6-Rg to the HBL-100 cell line showed that the interaction of CD6-Rg with this cell line was dose dependent and saturable (100  $\mu$ g/ml, Fig. 9A). Treating these cells with trypsin abolished CD6-Rg binding (Fig. 9B), while neuraminidase or N-glycanase treatment only slightly decreased CD6-Rg binding. The binding of CD6-Rg to HBL-100 was in part EDTA sensitive when the chelator was added before the CD6-Rg/HBL-100 binding, but not after (Fig. 10). In addition CD6-Rg bound very weakly to this cell line in PBS; however, addition of either  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  (2 mM final concentration) resulted in strong CD6-Rg binding to HBL-100 cells.

The ability of the anti-CD6 mAb MGB6 and G3-6 to block the binding of CD6-Rg to HBL-100 cells was examined. The MGB6 mAb was able to block CD6-Rg binding to HBL-100 in a concentration-dependent manner (Fig. 11), while the G3-6 mAb was unable to block CD6-Rg binding to HBL-100 cells at concentrations as high as 0.02 mg/ml (Fig. 11).

*Effects of cytokines on CD6-Rg binding.* Cytokines regulate the expression of a number of cell surface proteins. The effect of cytokines on the expression of the CD6-binding protein(s) expressed by HBL-100 cells was examined. Treatment of the cells with a mixture of

IL-1 $\beta$ , TNF $\alpha$ , and IFN- $\gamma$  resulted in the downregulation of CD6 ligand(s) expression (Fig. 12). Treatment with each of the cytokines alone or with pairwise combinations of the cytokines showed that a mixture of TNF $\alpha$  and IFN- $\gamma$  was predominantly responsible for the downregulation of CD6 ligand(s) expression by these cells.

*Immunoprecipitation of a CD6 ligand.* The CD6-Rg fusion protein was used to immunoprecipitate the CD6 ligand(s) expressed by HBL-100 cells. Multiple attempts to immunoprecipitate the CD6 ligand(s) following  $^{125}\text{I}$  cell surface labeling or [ $^{35}\text{S}$ ]methionine/cysteine metabolic labeling from both cell lines were unsuccessful. In contrast, [ $^3\text{H}$ ]glucosamine labeled surface glycoproteins of ~90 and ~40 kDa were immunoprecipitated from HBL-100 cell lysates with CD6-Rg (Fig. 13). It is unclear if the ~40-kDa species is a unique protein or a degradation product of the ~90-kDa protein. Similarly, CD6-Rg was able to immunoprecipitate proteins of ~90 and ~40 kDa from [ $^3\text{H}$ ]glucosamine-labeled H3606, a melanoma-derived cell line. CD6-Rg was unable to immunoprecipitate these proteins if EDTA was present in the cell lysate.

In addition, CD6-Rg but not CTLA4-Ig immunoprecipitated a polypeptide with a molecular mass of ~100 kDa from radiolabeled HBL-100 cell lysates in both the presence and the absence of EDTA (Fig. 13). This observation is consistent with cell binding studies which showed that CD6-Rg binding to HBL-100 cells was not completely blocked by EDTA (Fig. 10) but was completely abolished by pretreating the HBL-100 cells



with trypsin (Fig. 9B). This ~100-kDa polypeptide was also immunoprecipitated from the H3606 lysates by the CD6-Rg fusion protein.

*CD6-Rg Immunohistochemistry.* To identify tissues which expressed high levels of CD6 ligand(s) a panel of tissue sections obtained from murine brain, skin, liver, kidney, heart, spleen, lymph node, thymus, and small intestine were examined for CD6-Rg binding. Reactivity was observed in the skin, lymph node, and thymus tissue sections (Fig. 14). In the skin bright punctate staining of the dermis as well as staining of hair follicles was observed (Fig. 14A); however, human IgG<sub>1</sub> also reacted with the hair follicle, suggesting that this interaction was not mediated by the CD6 portion of the molecule. In the thymus the predominant reactivity was observed in the cortex (Fig. 14C) while in the lymph node bright staining was seen along the intermediate sinus (Fig. 14E).

Based on these observations, the binding of CD6-Rg to the murine thymic epithelial-derived cell line Z210 and to cultured human epidermal keratinocytes (EK) was examined. It was found that CD6-Rg bound to both Z210 and EK cells (Fig. 15). Binding to Z210 cells was trypsin sensitive, divalent cation-dependent, and modulated by cytokines in a similar manner to that observed with HBL-100 cells. The control fusion protein CD5-Rg did not bind to Z210 or EK cells (Fig. 15).

EXAMPLE IIIExperimental details:

Cells and culture conditions. TE cells and thymic fibroblasts (TF) were cultured by an explant technique as described (Singer et al, Human Immunol. 13:161 (1985); Singer et al, J. Invest. Dermatol. 92:166, (1989)). Human thymus tissue was obtained from the Department of Pathology, Duke University Medical Center, as discarded tissue from children undergoing corrective cardiovascular surgery, and thymocytes prepared as described (Denninget al, J. Immunol. 139:2573 (1989)). COS-M6 cells (ATCC, Rockville, MD) and HBL-100 cells (ATCC) were grown in DMEM containing 10% FCS, 1mM sodium pyruvate, 0.025 µg/ml amphotericin B, 100 u/ml penicillin and 100 µg/ml streptomycin.

Monoclonal antibodies. Antibodies used in this study were: P3X63/Ag8 (control mAb; ATCC), 35.1 (anti-CD2; from J. Hansen, Seattle, WA), T12 (anti-CD6; from E. Reinherz, Boston, MA), Nal/34 (anti-CD1a; from A. McMichael, Oxford, England), A3D8 (anti-CD44; (Telen et al, J. Clin. Invest. 71:1878 (1983)), J4-81 and J3-119 (Pesando et al, J. Immunol. 137:3689 (1986)), phycoerythrin conjugated anti-CD4 (CD4-PE; Dako, Carpinteria, CA) cychrome conjugated anti-CD8 (CD8-Cy; Pharmingen, San Diego, CA), and the blind panel of 479 mAbs from the 5th International Workshop on Human Leukocyte Differentiation Antigens (Shaw, Concepts of cross-lineage (blind panel) analysis of expression of

differentiation antigens. In: Leukocyte Typing, Schlossman et al, eds, Oxford University Press, Oxford (1994)).

*Detection of cell surface antigens.* Unfixed, 5 cultured TE cells, TF, COS cells or HBL-100 cells were suspended in either PBS, TBS (0.9 M NaCl, 50 mM Tris-HCl pH 7.3; with or without 5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, or 5 mM MnCl<sub>2</sub>) or DMEM (with or without 10 mM EDTA) containing 2% BSA and 0.1% NaN<sub>3</sub>. Cells were incubated with CD6-Rg 10 (Wee et al, Cell. Immunol. (1994)) or with CD5-Rg (Arrufo et al, Proc. Natl. Acad. Sci. USA 89:10242 (1992)), ELAM-Rg (Walz et al, Science 250:1132 (1990)), CTLA4-Rg recombinant fusion protein (Linsley et al, J. Exp. Med. 174:561 (1991)), or human IgG (Sigma, St. 15 Louis, MO) as controls (100 µg/ml) for 30 min at 4°C and washed with PBS containing 2% BSA and 0.1% NaN<sub>3</sub>. Fluorescein conjugated goat anti-human IgG<sub>1</sub> (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was used as a secondary reagent. Cells were analyzed on either a 20 Prifile II flow cytometer (Coulter Corp., Hialeah, FL) or a FACStar<sup>plus</sup> flow cytometer (Becton-Dickinson, Inc., Mountain View, CA) and data was processed using the software program PC-Lysys. To determine trypsin sensitivity of fusion protein interactions, cells were 25 incubated with 0.2% trypsin in PBS containing 1 mM EDTA for 30 min at 37°C and washed extensively prior to reactivity with fusion proteins. Three color immunofluorescence studies were performed.

*TE-thymocyte rosette binding assay.* TE-thymocyte rosette binding assays were performed as described (Singer et al, Proc. Natl. Acad. Sci. USA 83:6588 (1986)). Thymocytes were separated into CD1<sup>+</sup> (immature, CD6<sup>lo</sup>) or CD1<sup>+</sup> (mature, CD6<sup>hi</sup>) subpopulations by indirect immunofluorescent staining with NaI/34 and goat anti-mouse IgG followed by fluorescence activated cell sorting using a FACStar<sup>Plus</sup> fluorescence activated cell sorter. Thymocyte subpopulations were >95% pure on reanalysis of sorted cells. Mabs used in assays of TE-thymocyte binding were used at or in excess of saturating binding titers.

*COS cell transfection and binding studies.* Stable lines of CD6-expressing COS cells were constructed by co-transfection of plasmid CD6-15 containing the CD6 gene under the control of a CMV promoter (Aruffo et al, J. Exp. Med. 174:949 (1991)) and the pSVneo plasmid containing the bacterial neomycin resistance gene driven by an SV40 promoter as described (Liao et al, J. Immunol. 151:6490, (1993)). COS cells expressing CD6 were identified in indirect immunofluorescence assays using mAb T12. CD6<sup>+</sup> and CD6<sup>-</sup> cells were cloned using a Becton-Dickinson FACStar<sup>Plus</sup> fluorescence activated cell sorter.

CD6-expressing COS cells (COS-CD6) and control COS cells transfected with pSVneo (COS-neo) were used in suspension binding assays with TE cells. To differentiate between COS cells and TE cells, TE cells were metabolically labelled with 1  $\mu$ M calcein AM (Molecular Probes, Eugene, OR) for 15 min at 37°C in PBS

prior to harvest. Calcein AM-labelled cells are fluorescent and can be differentiated from non-labelled cells by fluorescence microscopy.

5        *Screening of mAbs for inhibition of CD6-Rg binding*  
to TE cells. Mabs from the blind panel of the 5th  
International Workshop on Human Leukocyte  
Differentiation Antigens and a panel of anti-integrin  
mabs were screened for reactivity to the surface of TE  
cells. Of the 154 mAbs that reacted with TE cells, 126  
10        were used in this assay. TE cells ( $10^5$ ) were incubated  
with ascites or purified mAb at a dilution of 1:100 for  
15 min at 4°C. Either CD5-Rg (5µg) or CD6-Rg (5µg) were  
added to this mixture and allowed to react for 2 hrs at  
4°C. After washing with PBS containing 2% BSA, CD5-Rg  
15        and CD6-Rg were labelled with a fluorescein-conjugated  
antiserum specific for the Fc portion of human IgG  
(Sigma). To account for any cross-reactivity with  
murine Ig that may have occurred with fluorescein-  
conjugated anti-human IgG, binding was determined as the  
20        difference in fluorescence ( $\Delta$ FL) between samples  
containing CD6-Rg and CD5-Rg. The  $\Delta$ FL of samples pre-  
incubated with control mAb P3 represented 100% binding.

25        *Antibody blocking studies.* MAb J4-81 was purified  
from ascites using a protein G-sepharose column (Pierce,  
Rockford, IL) and biotinylated with sulfo-NHS-biotin  
(Pierce) as recommended by the manufacturer. Biotin-J4-  
81 (1 µg/ml) was bound to the surface of TE cells in the  
presence of variable doses of mAbs A3D8, J4-81 or J3-  
119. After incubation with FITC-conjugated streptavidin

(Southern Biotechnology Associates, Inc., Birmingham, AL), cells were washed, fixed with 0.4% paraformaldehyde and analyzed by flow cytometry.

*Immunoprecipitation and protein labelling*

5 conditions. TE cells were metabolically labelled with 120  $\mu$ Ci/ml  $^3$ H-glucosamine (New England Nuclear, Boston, MA), harvested and immunoprecipitated as previously described (Wee et al, Cell. Immunol. (1994)). TE cell surface proteins were labelled with  $^{125}$ I (New England  
10 Nuclear) using lactoperoxidase as previously described (Jones, Analysis of radiolabeled lymphocyte proteins by one- and two-dimensional polyacrylamide gel electrophoresis. In: Selected methods in cellular immunology. Mishell and Shiigi, eds. W.H. Freeman and  
15 Company, New York, pp. 398-440 (1980)).

*Protein cross-linking.* TE cells, surface labelled with  $^{125}$ I, were incubated with mAbs (1:100 ascites), purified immunoglobulin (200  $\mu$ g/ml) or recombinant immunoglobulin fusion proteins (200  $\mu$ g/ml) for 2-4 hours  
20 in DME/5% FBS. After extensive washing with cold PBS, bound immunoglobulins were cross-linked to cell surface proteins with 1 mM DTSSP (Pierce) in PBS for 60 min at 4°C. DTSSP was inactivated with 20 mM Tris-HCl (pH 8.0) and the cells were washed with cold PBS. Cells were  
25 lysed in PBS containing 1% NP-40, 1 mM PMSF, 0.1 mM TLCK and 0.1%  $\text{NaN}_3$ . Immunoglobulin complexes were purified with protein A-sepharose beads (Sigma). Prior to SDS-PAGE, protein complexes were solubilized with SDS loading buffer (2% SD, 10 mM Tris-HCl pH 7.4, 20%

glycerol, bromphenol blue) containing 2% 2-mercaptoethanol (2-ME) to cleave the cross-linker. To confirm identity of proteins, purified protein complexes were treated with 0.2% trypsin in 1 mM EDTA for 30 min at 25°C (Patel et al, Virology 149:174 (1986)) prior to solubilization and cross-linker cleavage. After electrophoresis of proteins in discontinuous SDS-polyacrylamide gels, the gels were fixed in 40% methanol, impregnated with Amplify<sup>®</sup> reagent (Amersham, Arlington Heights, IL) dried and exposed to autoradiography film or imaged using a PhosphorImager System (Molecular Dynamics, Sunnyvale, CA).

*Immunohistology.* Normal human tissues were obtained as discarded tissue from the Department of Pathology, Duke University Medical Center and frozen in liquid nitrogen. Indirect IF assays of mAb reactivity on acetone fixed tissue sections were performed as described (Haynes et al, J. Invest. Dermatol. 76:323 (1982)).

## Results:

*Effect of anti-CD6 antibodies and CD6-Rg on TE-thymocyte binding.* The role of CD6 in the binding of thymocytes to TE cells was determined using a TE-thymocyte suspension binding assay. Both antibody to CD6 (T12) and recombinant CD6-immunoglobulin fusion protein (CD6-Rg) inhibited the binding of thymocytes to TE cells (Figure 16). As previously reported (Patel and Haynes, Semin. Immunol. 5:283 (1993)), anti-CD2 mAb 35.1

(as a positive control) inhibited TE-thymocyte binding by  $76 \pm 5\%$  ( $p < 0.003$ ). Anti-CD6 antibody T12 inhibited TE-thymocyte binding by  $49 \pm 9\%$  ( $p < 0.015$ ). Similarly, the CD6 human Ig fusion protein, CD6-Rg, inhibited TE-thymocyte binding by  $35 \pm 9\%$  ( $p < 0.05$ ). Addition of a combination of saturating amounts of mAbs 35.1 and T12 to TE-thymocyte binding assays resulted in a level of inhibition of binding ( $74 \pm 10\%$  inhibition) that was not significantly different from blocking by mAb 35.1 alone. Nonetheless, the data with anti-CD6 mAb and CD6-Rg suggested that CD6 may be an adhesion molecule that participates in the binding of thymocytes to TE cells, and that there may be a ligand for CD6 on human TE cells.

*Binding of CD6-expressing COS cells to TE cells.*

Although inhibition of TE-thymocyte binding with both anti-CD6 antibody and CD6-Rg was suggestive that CD6 was an adhesion molecule, inhibition of TE-thymocyte binding may have occurred because of steric hinderance. To confirm that CD6 was an adhesion molecule, stable transfectants of CD6-expressing COS cells (COS-CD6) were constructed (Figure 17). COS cells transfected with pSVneo only (COS-neo) did not bind well to TE cells ( $6 \pm 1\%$  COS-neo binding TE cells), whereas COS-CD6 did bind to TE cells ( $25 \pm 2\%$  COS-CD6 binding TE cells,  $p < 0.01$ ) (Figure 17). The specificity of the COS-CD6/TE binding was examined by testing for the inhibition of COS-CD6/TE binding by CD6 mAb T12. Compared to control mAb P3 and COS-neo cells ( $6 \pm 1\%$  COS-neo binding TE), CD6 mAb T12 completely inhibited the binding of COS-CD6



to TE cells ( $5 \pm 1\%$  COS-CD6 binding TE,  $p < 0.01$ ) to baseline levels of binding (Figure 17), confirming that COS-CD6/TE binding was CD6-specific.

5        *Effect of anti-CD6 mAb on the binding of thymocyte subsets to TE cells.* The role of CD6 in the binding of subsets of thymocytes was determined by examining the expression of CD6 on thymocyte subsets, and by testing the binding of sorted mature versus immature thymocyte subsets to TE cells in the presence of CD6 mAbs.

10       Expression of CD6 on thymocyte subsets was determined by three color immunofluorescence and flow cytometry (Figure 18). While all thymocytes expressed CD6, immature CD4+CD8+ double positive (DP) thymocytes were CD6<sup>lo</sup> and the mature CD4+CD8- or CD4-CD8+ single positive (SP) thymocytes were CD6<sup>hi</sup>.

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To determine the role of CD6 in the binding of thymocyte subsets to TE cells, thymocytes were separated into CD1+ and CD1- subsets by fluorescence activated cell sorting. CD1 was chosen because CD1+ thymocytes are the DP CD6<sup>lo</sup> cells and CD1- thymocytes are the SP CD6<sup>hi</sup> cells, and CD1 mAbs do not inhibit TE-thymocyte binding (Patel and Haynes, Semin. Immunol. 5:283 (1993)). Both CD1+ and CD1- thymocytes bound well to TE cells ( $45 \pm 4\%$  and  $54 \pm 6\%$ , respectively,  $p = \text{NS}$ ). CD2 mAb 35.1

20       inhibited the binding of immature thymocytes ( $77 \pm 9\%$  inhibition) to a greater degree than that of mature thymocytes ( $52 \pm 11\%$  inhibition) ( $p < 0.01$ ). CD6 mAb T12 also inhibited the binding of both immature ( $22 \pm 7\%$  inhibition) and mature ( $39 \pm 17\%$  inhibition) thymocytes

25       to TE cells ( $p < 0.25$ ) (Figure 19).

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*CD6-Rg binding to cells of the thymic*

*microenvironment.* The ability of CD6-Rg fusion protein to bind to TE cells, thymic fibroblasts and to thymocytes was determined by indirect IF and flow

5: cytometry. CD6-Rg bound to the surface of TE cells as well as to thymic fibroblasts, but not to thymocytes (Figure 20A). Binding of CD6-Rg to TE cells was

trypsin-sensitive and partially dependent upon divalent cations (Figure 20B). CD6-Rg bound well to TE cells in  
10 a buffer containing DME and 5% FBS, but in the presence of 10 mM EDTA, binding of CD6-Rg to TE cells was

inhibited by  $54 \pm 4\%$  (N=5,  $p < 0.001$ , Figure 20B). To further evaluate the divalent cation-dependence of CD6-Rg binding, CD6-Rg binding was determined in buffers  
15 containing 5 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$ , or 5 mM  $\text{MnCl}_2$ . While both  $\text{CaCl}_2$  and  $\text{MnCl}_2$  enhanced the binding of CD6-Rg to TE cells ( $144 \pm 5\%$ ,  $p = 0.01$ , and  $318 \pm 22\%$ ,  $P < 0.01$ , respectively),  $\text{MgCl}_2$  did not affect CD6-Rg binding ( $94 \pm 2\%$ ,  $p = \text{NS}$ ).

20 *Antibody-mediated inhibition of CD6-Rg binding to TE cells.* To begin to identify the CD6 ligand(s), a panel of 479 mAbs from the 5th International Workshop on Human Leukocyte Differentiation Antigens was screened for reactivity to the surface of TE cells by indirect  
25 immunofluorescence and flow cytometry (Shaw, Concepts of cross-lineage (blind panel) analysis of expression of differentiation antigens. In: Leukocyte Typing, Schlossman et al, eds, Oxford University Press, Oxford (1994)). Of the 154 mAbs that reacted with the surface  
30 of TE cells, 126 mAbs were used in assays to inhibit the

binding of CD6-Rg to TE cells. Of the 122 mAbs that did not react with the secondary antiserum, only one (J4-81) inhibited the binding of CD6-Rg to TE cells. MAb J4-81 inhibited the binding of CD6-Rg to TE cells by  $60 \pm 7\%$  (N=10,  $p < 0.001$ ) and to the breast cell line HBL-100 by  $40 \pm 3\%$  (N=3,  $p < 0.02$ ) (Table 4), which has also been shown to bind CD6-Rg (Wee et al, Cell. Immunol. (1994)). In flow cytometry assays, both TE cells and HBL-100 cells reacted strongly with mAb J4-81.

Table 4. MAb inhibition of CD6-Rg binding to TE and HBL-100 cells.

Cell Type	mAb	$\Delta$ Fluorescence*	% Binding <sup>‡</sup>	% Inhibition <sup>§</sup>
TE cells	P3	52	100	0
	CD9	60	115	-15
	CD24	48	92	8
	CD40	48	92 <sub>1</sub>	8
	CD46	57	110	-10
	CD51	46	88 <sub>1</sub>	12
	CD54	44	85	15
	CD58	45	87	13
	CD59	52	100	0
	CD63	53	102	-2
	CD66	56	108	-8
	J4-81	21	40	60
HBL-100	P3	58	100	0
	J4-81	34	59	41

\* $\Delta$ Fluorescence = Fluorescence(CD6-Rg) - Fluorescence(CD5-Rg). Shown is the binding of CD6-Rg compared to control (CD5-Rg) in the presence of selected mAbs that bind well to the surface of TE cells.

<sup>‡</sup>% Binding =  $100[\Delta\text{FL}(\text{experimental mAb}) - \Delta\text{FL}(\text{P3})]/\Delta\text{FL}(\text{P3})$ .

<sup>§</sup>% Inhibition =  $100[\Delta\text{FL}(\text{P3}) - \Delta\text{FL}(\text{experimental mAb})]/\Delta\text{FL}(\text{P3})$ .

MAB J3-119, reported to react with a second epitope on the molecule detected by J4-81 (Pesando et al, J. Immunol. 137:3689 (1986)), enhanced the binding of CD6-Rg to TE cells (Figure 21) by  $48 \pm 5\%$  ( $N=6$ ,  $P<0.005$ ), and to HBL-100 cells by  $45 \pm 11\%$  ( $N=3$ ,  $p<0.1$ ). To confirm that mAb J3-119 recognized the same protein as mAb J4-81, the ability of mAb J3-119 to block the binding of biotinylated J4-81 to TE cells was tested. While mAb A3D8 to CD44 (which binds well to the surface of TE cells had no effect on J4-81 binding, both J4-81 and J3-119 mAbs inhibited the binding of biotinylated J4-81 (99% and 82%, respectively) (Figure 22). Moreover, mAb J4-81 modulated surface expression on B cells of the antigen detected by J3-119 (Pesando et al, J. Immunol. 137:3689 (1986)).

To further establish that mAb J4-81 recognized a ligand for CD6, the ability of J4-81 to inhibit the CD6-specific binding of COS-CD6 cells to TE cells was tested. MAb J4-81 significantly inhibited the binding of TE cells to COS-CD6 cells ( $87 \pm 1\%$ ,  $N=3$ ,  $p<0.001$ ), thus confirming that J4-81 recognized a CD6 ligand.

*MAB J4-81 and CD6-Rg bind 100 kDa TE cell proteins that are identical.* To identify the TE cell surface protein(s) that bound to CD6-Rg, a strategy was devised whereby CD6-Rg interactions with CD6 ligand(s) on surface  $^{125}\text{I}$  labelled TE cells were stabilized with DTSSP, a cleavable homobifunctional cross-linking reagent reactive with free amino groups, and CD6-Rg containing complexes were purified using protein A-sepharose beads. Using this strategy, CD6-Rg

specifically reacted with a 100 kDa TE cell surface protein (Figure 23). MAb J4-81 cross-linked to  $^{125}\text{I}$  labelled TE cells also yielded an 100 kDa protein that migrated with the 100 kDa protein recognized by CD6-Rg (Figure 23). Trypsin digestion studies showed that the 100 kDa protein identified by J4-81 had an identical trypsin digestion pattern to the 100 kDa protein identified by CD6-Rg (Figure 23), indicating that both proteins were identical. MAb J4-81 also specifically immunoprecipitated an 100 kDa protein from extracts of TE cells metabolically labelled with  $^3\text{H}$ -glucosamine.

*The 100 kDa CD6 ligand is a divalent cation-independent ligand for CD6.* The immunoprecipitation studies (above) showed that CD6-Rg was able to immunoprecipitate a 100 kDa protein in the presence and absence of divalent cations. Thus, the 100 kDa protein may be a divalent cation-independent CD6 ligand. Further, J4-81 only partially inhibited the binding of CD6-Rg to TE cells in the presence of divalent cations, suggesting that there may be more than one ligand for CD6. MAb J4-81 nearly completely inhibited ( $80 \pm 10\%$  inhibition,  $p < 0.1$ ) CD6-Rg binding to TE cells in the presence of EDTA (Figure 21), confirming that the protein recognized by JA-81 is primarily involved in divalent cation-independent CD6-CD6 ligand interactions. In contrast, mAb J3-119 enhanced CD6-Rg binding by  $47 \pm 7\%$  ( $p < 0.1$ ) in the absence of divalent cations (Figure 21).

*Tissue distribution of mAb J4-81 reactivity.* The tissue distribution of the 100 kDa glycoprotein recognized by J4-81 was examined on frozen sections of a variety of human tissues by indirect IF (Table 5). The reactivity of mAb J4-81 with human tissues and cell lines was broad. While CD6 was expressed on thymocytes in postnatal human thymus, the 100 kDa CD6 ligand was expressed on cortical and medullary TE cells and Hassall's bodies (Figure 24). In tissues other than thymus, J4-81 reacted with epidermal keratinocytes, gut epithelium, breast epithelium, pancreatic acinar and islet cells, hepatocytes, renal tubular epithelium, neurons of the cerebral cortex, and fibroblasts.

Table 5 Reactivity of mAb J4-81 in sections of normal human tissues.

Tissue (Number Tested)	J4-81 Reactivity
Thymus (5)	Hassall's bodies, epithelium, fibroblasts
Spleen (2)	Scattered mononuclear cells
Lymph Node (1)	Scattered mononuclear cells
Tonsil (2)	Pharyngeal epithelium, lymphocytes
Appendix (1)	Fibroblasts, lymphoid cells, epithelium
Colon (2)	Fibroblasts, epithelium
Esophagus (2)	Basal epithelium
Breast (2)	Epithelium, fibroblasts
Liver (2)	Hepatocytes, Kupfer cells, fibroblasts
Pancreas (2)	Acinar and islet cells
Kidney (2)	Fibroblasts, subset of tubules, Bowman's capsule
Skin (2)	Perivascular fibroblasts, epithelium
Brain (2)	Neurons

The cell types reacting with mAb J4-81 in each of the tissue types are listed.



EXAMPLE IVExperimental details:

*Cells lines, tissue culture, antibodies.* TE cells were cultured by an explant technique as described (Singer et al, Human Immunol. 13:161 (1985); Singer et al, J. Invest. Dermat. 92:166, (1989)). COS cells, breast carcinoma HBL-100, B cell lymphoma cell lines Ramos, Raji and Daudi, T cell lymphoma cell lines CEM and MOLT4, erythroleukemia cell line K562, and monocyte-like cell lines HL60 and U937 were obtained from ATCC (Rockville, MD) and maintained under standard tissue culture conditions in Iscove's Modified Dulbecco's Medium plus 10% fetal bovine serum. NK-like cell line YT was obtained from E. Podack (Univ. of Miami Sch. of Medicine). Antibodies used in this study were J4-81 (Pesando et al, J. Immunol. 137:3689 (1986)) as ascites, Leu4 (anti-CD3)-phycoerythrin (Becton Dickinson, San Jose, CA), G3-6 (anti-CD6 from J. Ledbetter, Bristol-Myers Squibb), anti-L6 (Bristol-Myers Squibb), T12 (anti-CD6, ATCC), and P3 (ATCC).

*Binding of TE cells to COS cell transfectants expressing CD6.* COS-CD6 and COS-neo cells were prepared by co-transfecting a plasmid encoding CD6 (Aruffo et al, J. Exp. Med. 174:949 (1991)), and the pSVneo plasmid (Liao et al, J. Immunol. 151:6490 (1993)). Following G418 selection, CD6+ cells were cloned using a Becton-Dickinson FACStar<sup>PLUS</sup> fluorescence activated cell sorter. COS-neo and COS-CD6 TE cells were used in a suspension

binding assay. To differentiate between COS cells and TE cells, TE cells were labeled with 1  $\mu$ M calcein AM (Molecular Probes, Eugene, OR) for 15 min. at 37° in PBS prior to harvest. Calcein AM-labeled cells are  
5 fluorescent and can be differentiated from unlabeled cells by fluorescence microscopy. Adhesion was quantitated by scoring the percentage of COS-CD6 cells that were conjugated with TE cells. For blocking adhesion, antibodies T12, J4-81, and P3 were used at or  
10 in excess of saturating binding titers.

*Cloning and characterization of the gene for the antigen recognized by mAb J4-81.* J4-81 immunoaffinity purification of ALCAM from HBL-100 cells ( $\sim 3 \times 10^9$ ) was performed as described (Bowen et al, J. Immunol.  
15 151:5896 (1993)). The purified protein and CNBr fragments were analyzed in a pulsed-liquid protein sequencer by previously described methods (Maresh et al, Arch. Biochem. Biophys. 311:95 (1994)). Amino-terminal sequences were screened against the SwissProt data base  
20 using Genetics Computer Group (GCG) sequence-analysis software. Chicken BEN probes were synthesized by PCR from a chicken embryo cDNA library (Clontech), random prime labeled with  $^{32}$ P-dCTP, and used to screen a PHA activated human T cell cDNA library. A partial cDNA  
25 clone was isolated; 200 bp of the 5' end of this cDNA was used to isolate a clone containing the entire coding region from a HL60 cDNA library. Database searches with the cDNA derived peptide sequence of ALCAM were performed using the BLAST program, alignments and  
30 consensus sequence derivation of the four highest

scoring hits were performed using the Pileup and Pretty programs from the GCG sequence analysis software.

*Northern analysis.* Peripheral blood mononuclear cells (PBMC) were isolated from whole blood with lymphocyte separation medium (Organon Teknika, Durham, NC) according to the manufacturer's instructions. PBMC were stimulated with 1  $\mu$ g/ml phytohemagglutinin (PHA; Boehringer Mannheim Indianapolis, IN). Total cellular RNA was prepared with TRIzol reagent (Gibco BRL, Gaithersburg, MD). Monocytes were isolated from PBMC's by adherence to plastic for 2.5h. The RNA's were fractionated on a denaturing formaldehyde gel; 25  $\mu$ g of RNA was loaded for all samples except for monocytes, where 15  $\mu$ g was loaded. RNA was transferred to nitrocellulose TC (Schleicher and Schuell, Keene, NH) and probed with a random primed  $^{32}$ P-dCTP labeled ALCAM or GAPDH cDNA. Filters were exposed to film at -70°C and developed.

*PHA blast preparation, staining.* PBMC were isolated and activated with PHA as described above and stained on selected days with mAb J4-81 ascites (1:200) or CD6-Rg (50  $\mu$ g/ml) followed by a FITC labeled second antibody and anti-CD3-PE; cytofluorometric analysis of J4-81 staining T cells was performed on a FACScan (Becton Dickinson, San Jose, CA) by gating on CD3 positive cells.

*Chromosomal mapping of the gene encoding ALCAM.* Two overlapping cDNA clones of the human ALCAM gene

containing 2.2 kb and 1.9 kb inserts in CDM7 were used as probes for fluorescence *in situ* hybridization (FISH) as described (Milatovich et al, Genomics 11:1040 (1991)). The probe was labeled with biotin-dUTP by  
5 nick-translation and was hybridized at a concentration of 500 ng/50 $\mu$ l per slide to pre-treated and denatured metaphase chromosomes from a human PHA-stimulated lymphocyte culture. Salmon sperm DNA served as carrier but no human genomic DNA was used as competitor. After  
10 incubation, washing, signal detection with avidin-FTIC (Vector Laboratories) and one round of amplification with biotinylated goat anti-avidin D antibody (Vector Laboratories), chromosomes were counterstained with propidium iodide and analyzed under an Axiophot (Zeiss)  
15 epifluorescence microscope as described (Milatovich et al, Genomics 11:1040 (1991)). Hybridization signals were considered specific only when the signal was observed on both chromatids of a chromosome. Results were recorded by digital imaging using a cooled charge-  
20 coupled device (CCD) camera (Photometrics PM512) and displayed in pseudo-colors.

*Construction of ALCAM-Rg.* ALCAM-Rg was constructed by PCR technology similarly to CD6-Ig (Aruffo et al, Proc. Natl. Acad. Sci. USA 89:2292 (1992)). ALCAM cDNA  
25 in pCDM8 was used as a template. A forward oligonucleotide primer (GGCCAGATATACGCGTTGACATT) encompassing an MluI site in the CMV promoter of pDCM8 and a reverse primer  
(TGTATCATGTGGATCCGCTGGTCATTACCTTTTCTCT) containing a  
30 BamHI site and specific for the nucleotides encoding the

extracellular membrane proximal residues of ALCAM were used to synthesize a fragment that encoded a DNA fragment encoding truncated ALCAM. This PCR product was digested with MluI and BAMHI and ligated to a vector (pCDM7) that was similarly digested and encoding the hinge, CH1 and CH2 domains of human IgG1. This resulted in a chimeric gene encoding the extracellular region of ALCAM fused to the Fc portion of human IgG1. Protein was produced by transient expression of ALCAM-Rg in COS cells and purified by absorption to and elution from protein A Sepharose. Briefly, plasmid DNA was transfected into COS cells by the DEAE-dextran chloroquine method. 18-24 hours later, the 10% FBS Dulbecco's modified Eagle's medium was replaced by serum-free media after a rinse wash with PBS. The cell culture was maintained 7-10 days before it was harvested. Briefly, the supernatant containing the fusion protein was centrifuged at 2500 rpm or 20 min and filtered through a 0.45  $\mu$ m Millipore filter. The supernatant was passed over a Protein A Sepharose column at a flow rate of 1.00 ml/min. The bound fusion protein was eluted with pH 3.5, 0.1 M citric acid and was immediately neutralized with pH 9.0, 1.0 M Tris. The fusion protein was dialyzed against PBS and concentrated with a Certicor concentrator (Amicon).

*Binding of CD6-Rg to ALCAM expressing COS cells and binding of ALCAM-Rg to CD6 expressing COS cells.* COS cells were either transfected with parent vector CDM8 or with cDNA clones encoding CD6 or ALCAM by the DEAE-dextran method (Aruffo et al, Proc. Natl., Acad., Sci.,

USA 84:8573 (1987)). Three days post-transfection the cells were detached with PBS/0.5 mM EDTA, washed with PBS, and resuspended in PBS/1% BSA/0.1% NaN<sub>3</sub> (PBA) containing the following concentrations of mAb's or fusion proteins: J4-81 (aALCAM) 1:200 dilution of ascites, G3-6 (aCD6) 5 µg/ml, CD6-Rg 50 µg/ml, and ALCAM-Rg 25 µg/ml. After 30 min. on ice, cells were washed twice with PBA and resuspended in PBA containing either goat anti-mouse or human IgG-FITC for 30 min. on ice. The cells were washed twice in PBA, once with PBS, fixed in PBS/1% formaldehyde, and analyzed by flow cytometry.

#### Results:

CD6 on transfected COS cells promotes TE cell adhesion. To examine directly the role of CD6 in thymocyte-TE cell adhesion, a stable COS cell transfectant expressing CD6 was prepared. COS cells expressing CD6 (COS-CD6) supported TE cell adhesion while COS cells transfected with the parent vector alone (COS-neo) did not (Fig. 25). The adhesion of TE cells to COS-CD6 cells was inhibited by pretreating the COS-CD6 cells with anti-CD6 mAb T12 or by pretreating TE cells with J4-81 mAb (Fig. 25B), indicating that CD6 mediated adhesion to TE cells involves the molecule recognized by mAb J4-81.

Cloning and characterization of the antigen recognized by mAb J4-81 (activated leukocyte-cell adhesion molecule (ALCAM)). A J4-81 affinity column was

used to purify the protein from HBL-100, a breast carcinoma cell line that expressed high levels of a CD6 ligand (Wee et al, Cell. Immunol. 158:353 (1994)) and the antigen recognized by J4-81 (Patel et al, J. Exp. Med. (1994)). Amino acid sequences obtained from intact protein and internal peptide fragments were found to have substantial homology with the chicken neural adhesion molecule BEN/SC-1/DM-GRASP (Pourquie et al, Proc. Natl. Acad. Sci. USA 89:5261 (1992)); Tanaka et al, Neuron 7:535 (1991)); Burns et al, Neuron 7:209 (1991)), which was also reported to be expressed by activated chicken leukocytes (Corbel et al, Cell Immunol. 141:99 (1992)). DNA fragments corresponding to chicken BEN were obtained by PCR and used to screen a PHA activated human T cell cDNA library. A single clone that did not contain the complete coding region was isolated. A 200 bp PCR fragment derived from the 5' region of this clone was used to isolate a ~1.9 kb cDNA clone that contained the complete coding sequence from an HL60 cDNA library (the nucleotide sequence has been deposited in GenBank, accession #L38608). The two cDNA clones exhibit polymorphisms at three nucleotide positions in the region of overlap, two of which result in differences at the protein level (G to A from HL60 to the T cell clone at position 836 results in the substitution of N231 by S; C to T from HL60 to the T cell clone at position 965 results in the substitution of M274 by T).

The predicted amino acid sequence of the human homologue of chicken BEN (ALCAM), a type I membrane protein, consists of a 27 amino acid (aa) N-terminal

hydrophobic signal peptide, followed by 500 aa extracellular domain, a 24 aa hydrophobic transmembrane domain, and a 32 aa cytoplasmic domain (see Figure 29). Glycosylation of ALCAM probably accounts for much of the difference between the predicted molecular weight of ~65 kDa and the 100-105 kDa molecular weight observed by immunoprecipitation (Patel et al, J. Exp. Med. (1994)); Pesando et al, J. Immunol. 137:689 (1986)). Comparison of the aa sequence of ALCAM with others in the data base showed that it was homologous to neurolin (Lawssing et al, Differentiation 56:21 (1994)), a protein expressed by neural axons of the goldfish visual system (38% identity/55% similarity), RAGE (Neeper et al, J. Biol. Chem. 267:14998 (1992)), a receptor for advanced glycation end products (28/43%), and MUC18 (Lehmann et al, Proc. Natl. Acad. Sci. USA 86:9891 (1989)), a cell surface protein whose expression correlates with the metastatic potential of melanoma cells (23/49%) (Fig. 26A).

*ALCAM is expressed by activated leukocytes.* By Northern blot analysis an ALCAM cDNA probe hybridized with a ~5.2 kb mRNA expressed by mitogen activated peripheral blood mononuclear cells (PBMC) and a number of T cell, B cell, monocytic, and tumor derived cell lines; activated monocytes showed two additional minor species of ~10 and 8.5 kb (Fig. 26B). ALCAM mRNA was not detected in unactivated PBMC. To examine if the presence of ALCAM transcripts in activated T cells corresponded to the expression of a J4-81 and CD6-Rg binding protein, PBMC were activated with PHA and



5 aliquots of the bulk culture were examined by flow cytometry for 10 days. Expression of a J4-81 and CD6 binding protein on T cells was observed two days after activation, was maximal at three days, and declined to undetectable levels eight days after activation (Fig. 26C). The binding of J4-81 and CD6-Rg to activated T cells exhibits the same expression kinetics, indicating that these two molecules recognize the same molecular target.

10           *Assignment of human ALCAM gene to chromosome bands 3q13.1-q13.2.* Fluorescence in situ hybridization experiments using ALCAM cDNA clones as probes localized the gene to a single site on the proximal long arm of human chromosome 3. Of 25 metaphase spreads analyzed,  
15 20 exhibited a specific fluorescent signal on both chromatids at this site, and 13 and 20 had signal on both chromosome 3 homologues. The chromosomes were identified by an R-banding pattern produced by the incorporation of BrdU after synchronization of the cell  
20 culture. The ALCAM signal was assigned to bands 3q13.1-q13.2 (Fig. 27).

25           *ALCAM is a CD6 ligand.* To directly examine if the ALCAM cDNA clone isolated from HL60 cells directs the expression of a protein which binds J4-81 and CD6-Rg, the cDNA was transfected into COS cells. Three days post-transfection, the COS cells were examined for their ability to bind to J4-81 (aALCAM) or CD6-Rg by flow cytometry. As shown in Fig. 28A, COS cells expressing ALCAM were able to bind to both J4-81 and CD6-Rg. To

further confirm that ALCAM is a CD6 ligand, a chimeric gene encoding ALCAM-Rg was constructed. This protein was expressed as a covalent homodimer with a molecular mass of ~200 kDa and was recognized by mAb J4-81. To  
5 demonstrate the binding of ALCAM-Rg to CD6, COS cells were transfected with a cDNA clone encoding CD6 (Aruffo et al, J. Exp. Med. 174:949 (1991)) and examined by flow cytometry three days later. COS cells transfected with CD6 bound both G3-6 (aCD6) and ALCAM-Rg (Fig. 28B).  
10 These results demonstrate that CD6 and ALCAM are a receptor/ligand pair.

Chicken BEN has been reported to mediate homotypic adhesion (Pourquie et al, Proc. Natl. Acad. Sci. USA 89:5261 (1992)); Tanaka et al, Neuron 7:535 (1991));  
15 Burns et al, Neuron 7:209 (1991)). ALCAM-Rg was able to bind COS cells that expressed ALCAM, indicating that ALCAM mediates both homophillic and heterophillic adhesion.

\* \* \*

20 All documents cited above are hereby incorporated in their entirety by reference.

One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true  
25 scope of the invention.

WHAT IS CLAIMED IS:

1. A purified CD6 ligand, or portion thereof of at least 5 consecutive amino acids.

2. The ligand according to claim 1 wherein the binding of said ligand to CD6 is divalent cation independent.

3. The ligand according to claim 1 wherein the binding of said ligand to CD6 is divalent cation dependent.

4. The ligand according to claim 1 wherein said ligand has the amino acid sequence shown in Figure 29.

5. The ligand according to claim 1 wherein said ligand is derivable from a thymic epithelial cell.

6. The ligand according to claim 5 wherein said thymic epithelial cell is a human cell.

7. An isolated CD6 ligand having a molecular weight of about 35 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions, or portion thereof of at least 5 consecutive amino acids.

8. An isolated CD6 ligand having a molecular weight of about 100 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis under

reducing conditions, or portion thereof of at least 5 consecutive amino acids.

9. An isolated CD6 ligand having a molecular weight of 90 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions, or portion thereof of at least 5 consecutive amino acids.

10. A composition comprising the CD6 ligand according to one of claims 1, 7, 8 or 9, and a pharmaceutically acceptable carrier.

11. A mimetope of the CD6 ligand according to one of claims 1, 7, 8 or 9.

12. The mimetope according to claim 11 wherein said mimetope is RNA, DNA or protein.

13. A composition comprising the mimetope according to claim 11 and a pharmaceutically acceptable carrier.

14. A method of inhibiting binding of CD6 present on the surface of a first cell to CD6 ligand present on the surface of a second cell comprising contacting said CD6 present on the surface of said first cell with a soluble CD6 ligand, or mimetope thereof, under conditions such that said soluble CD6 ligand, or mimetope thereof, binds to said CD6 present on the surface of said first cell and thereby inhibits binding

of said CD6 ligand present on the surface of said second cell to said CD6 present on the surface of said first cell.

15. A method of inhibiting binding of CD6 present on the surface of a first cell to CD6 ligand present on the surface of a second cell comprising contacting said CD6 ligand with soluble CD6, or mimetope thereof, under conditions such that said soluble CD6, or mimetope thereof, binds to said CD6 ligand present on the surface of said second cell and thereby inhibits binding of said CD6 present on the surface of said first cell to said CD6 ligand present on the surface of said second cell.

16. The method according to one of claims 14 and 15 wherein said first cell is a T cell, B cell or neuronal cell, and said second cell is a thymic epithelial cell, a B cell, a monocyte/macrophage, an epithelial cell, a pancreatic acinar cell, a pancreatic islet cell, a fibroblast or a neuronal cell.

17. A method of screening test compounds for the ability to inhibit binding of CD6 to CD6 ligand comprising:

- i) contacting a test compound with one of CD6 or CD6 ligand, or respective binding portion thereof, under conditions such that a complex between said test compound and said CD6 or CD6 ligand can form;
- ii) contacting the other of said CD6 or CD6 ligand with the combination resulting from step (i) under conditions such that binding of said CD6, or respective

binding portion thereof, to said CD6 ligand, or respective binding portion thereof can occur; and

iii) determining the extent of binding of said CD6, or respective binding portion thereof, to said CD6 ligand, or respective binding portion thereof, and thereby the ability of said test compound to inhibit binding of said CD6 to said CD6 ligand.

18. The method according to claim 17 wherein at least one of said CD6 or CD6 ligand, or respective binding portion thereof, is in soluble form.

19. The method according to claim 17 wherein at least one of said CD6 and CD6 ligand is present on the surface of a cell.

20. An antibody to an approximately 90 kDa (as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions) form of a CD6 ligand.

21. An isolated nucleic acid molecule having a sequence that encodes CD6 ligand, or at least a 5 amino acid portion thereof.

22. The molecule according to claim 21 wherein said molecule is a cDNA molecule.

23. An expression construct comprising the molecule according to claim 21 and a vector, wherein

said molecule is operably linked, in said vector, to a promoter.

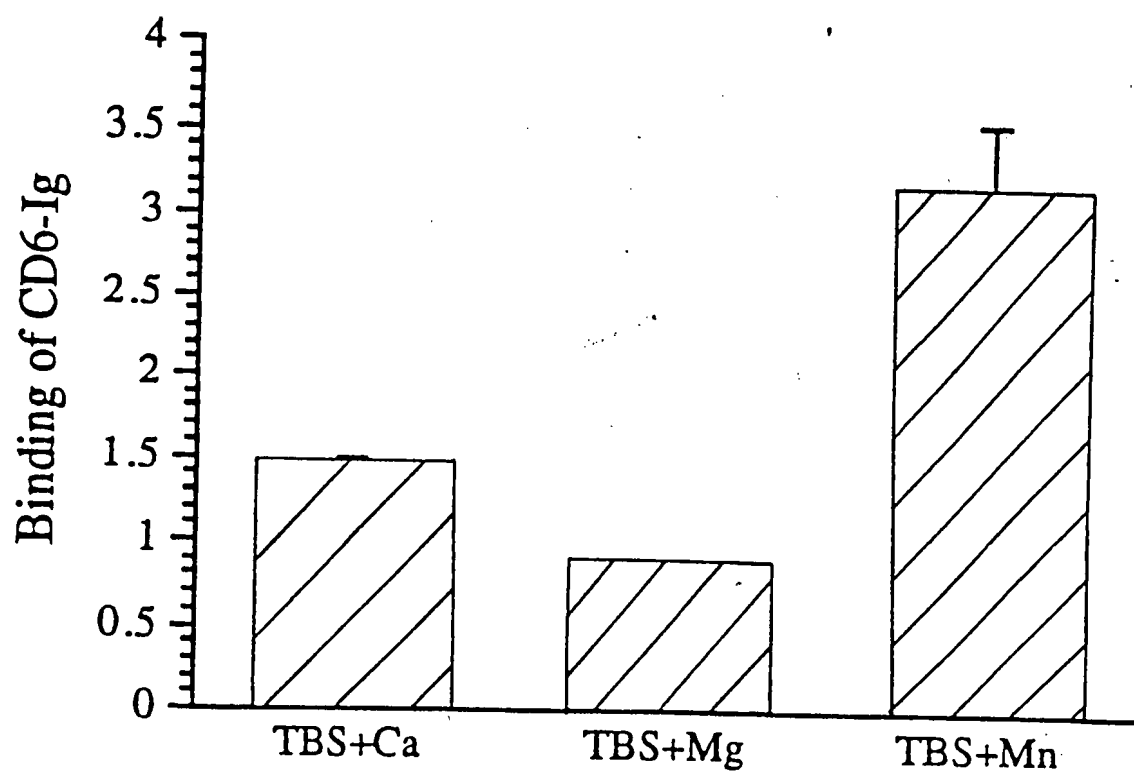
24. The molecule according to claim 21 wherein said CD6 ligand has the sequence shown in Figure 29.

25. The molecule according to claim 24 wherein said molecule has the sequence shown in Figure 29.

26. A host cell comprising the expression construct according to claim 23.

27. A method of producing a CD6 ligand or portion thereof comprising culturing the host cell according to claim 26 under conditions such that said nucleic acid molecule is expressed and said CD6 ligand is thereby produced.

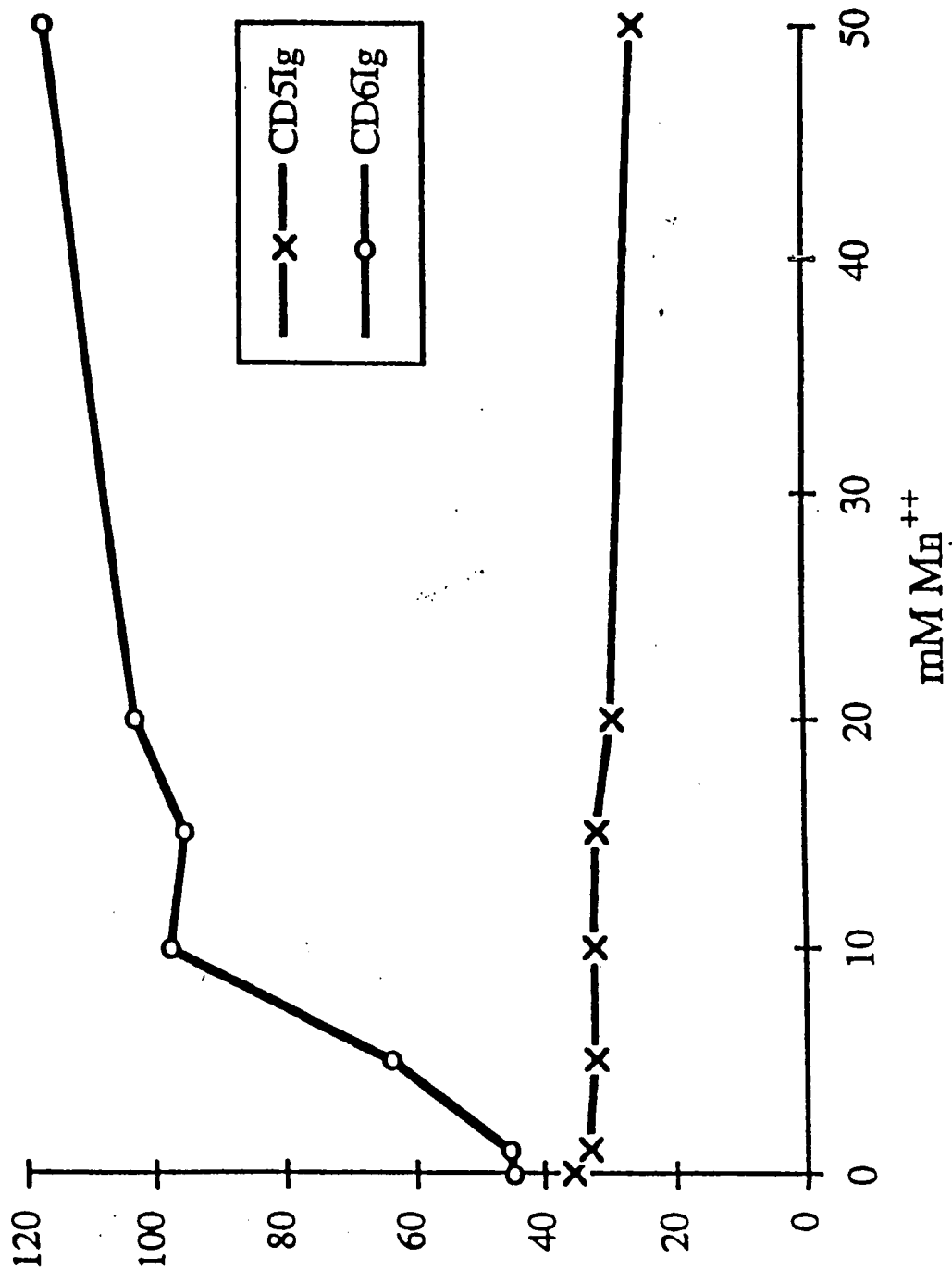
1/30

**FIG. 1**



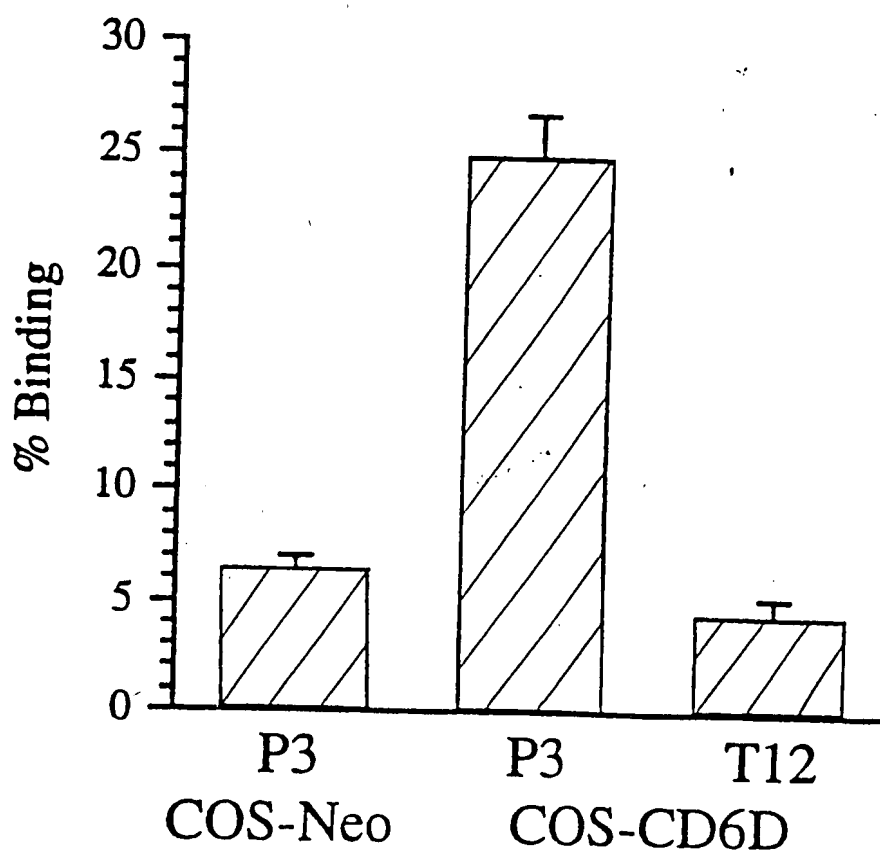
2/30

FIG. 2

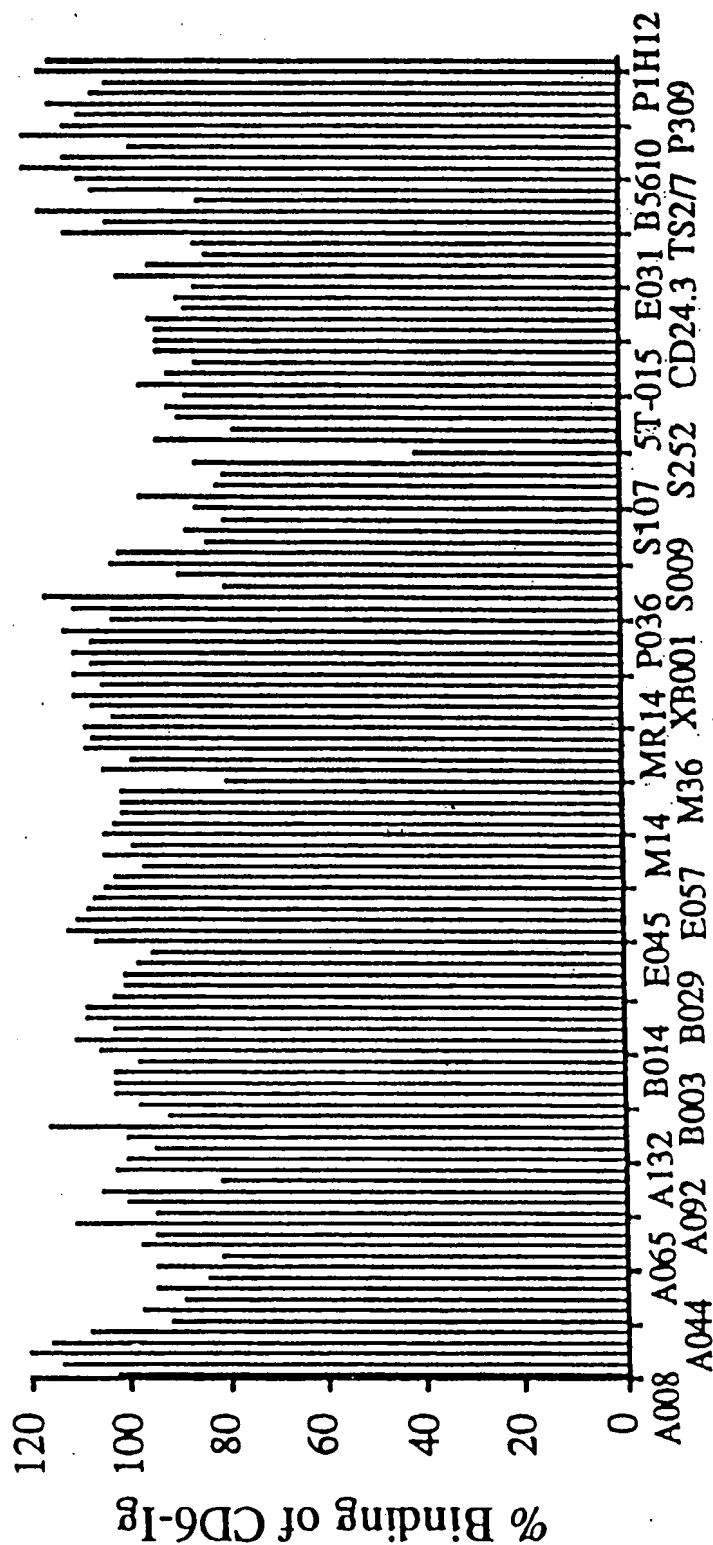


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**FIG. 3**

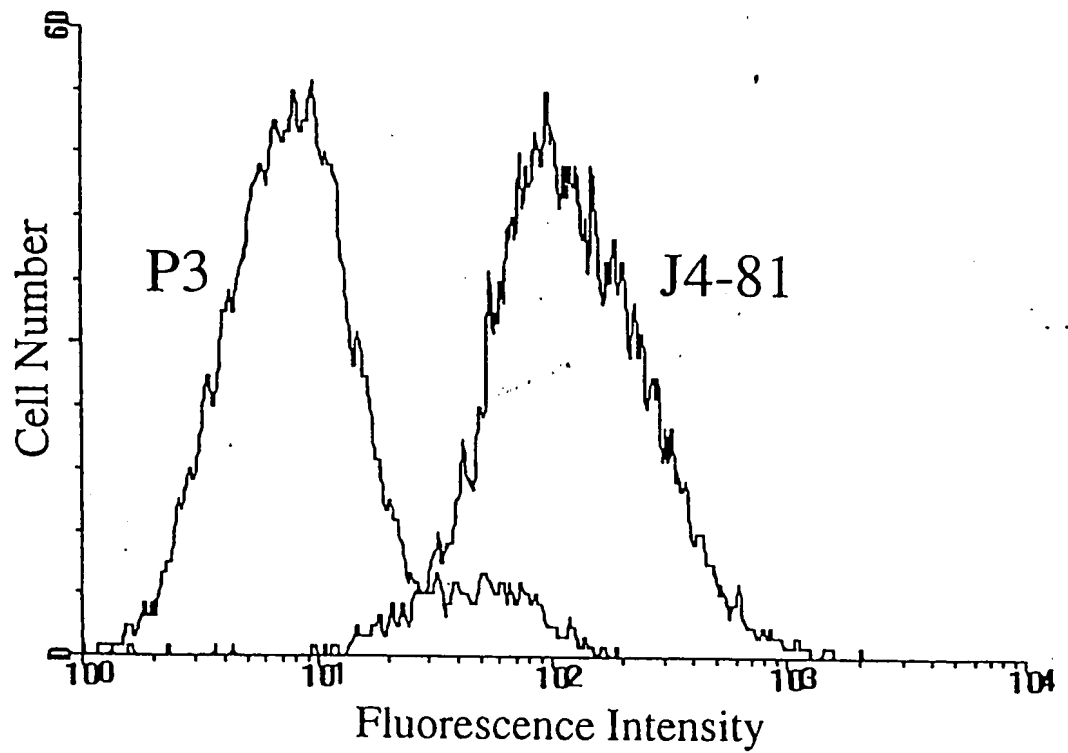
4/30



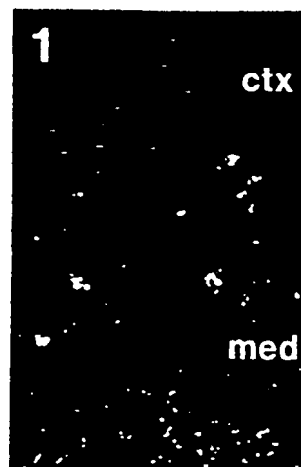
monoclonal antibodies

**FIG. 4**

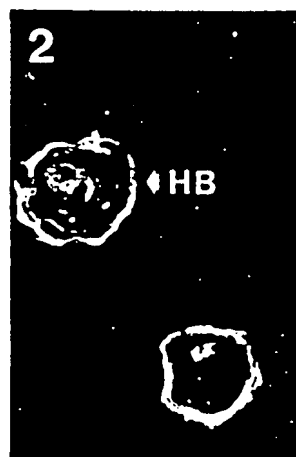
5/30

**FIG. 5**

6/30



**FIG. 6A**

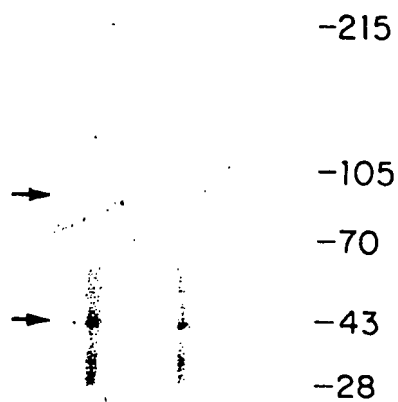


**FIG. 6B**

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CD6-Rg  
CD6-Rg+EDTA  
CTLA4-Ig



**FIG. 7**

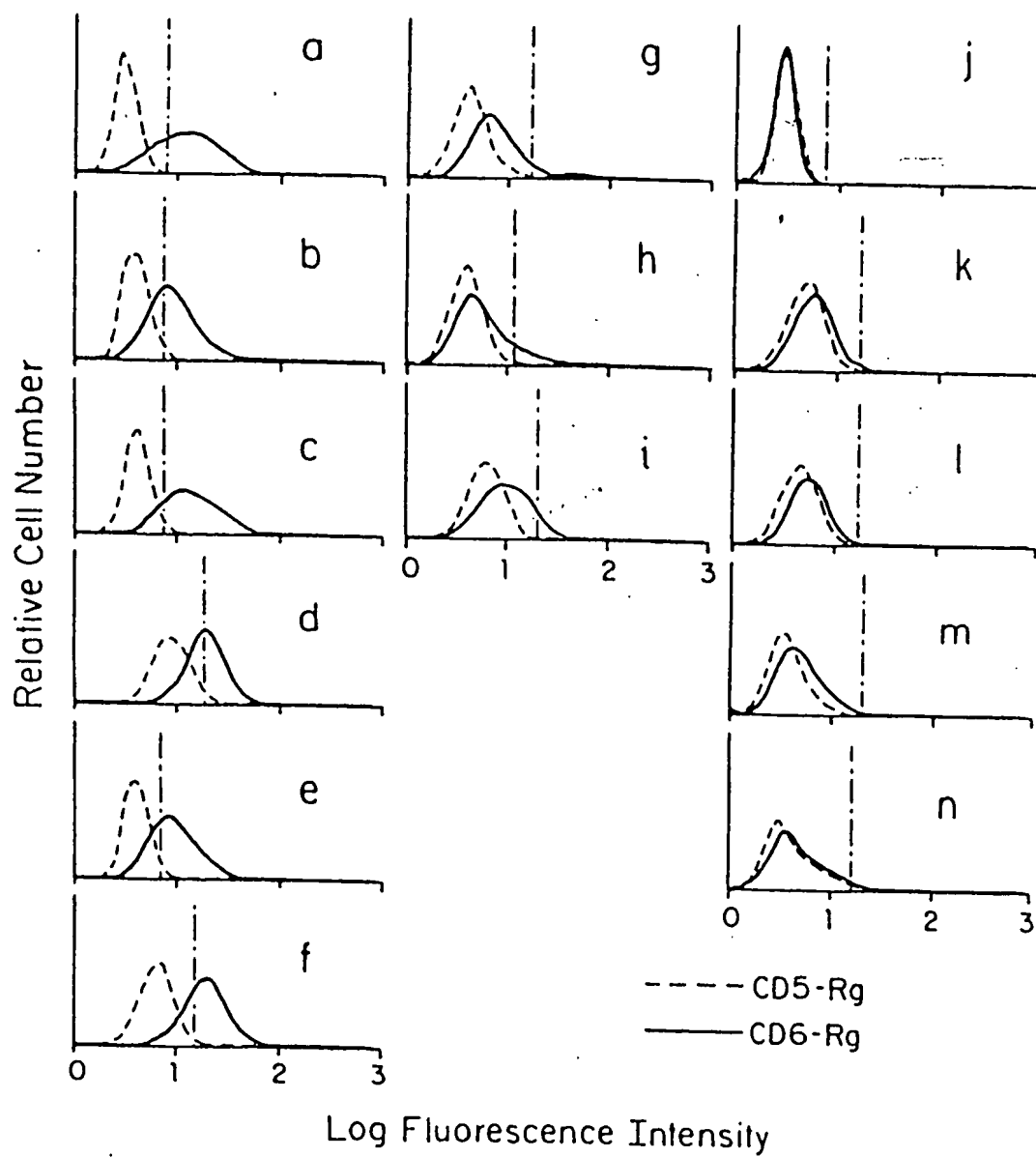
8/30

## CHARACTERIZATION OF THE CD6 LIGAND(S)

High Binding  
( >50%)

Low Binding  
( $<20\%$ )

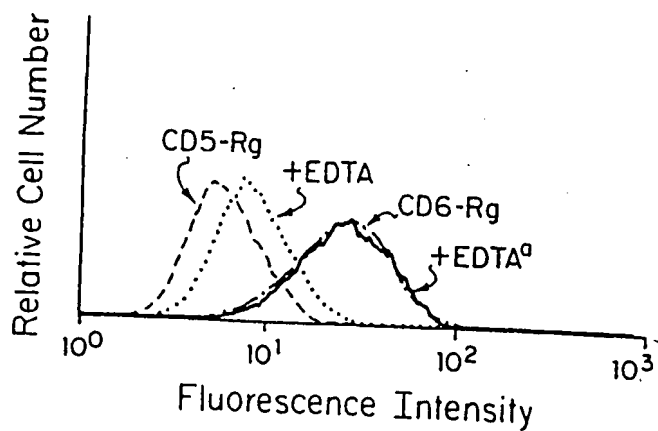
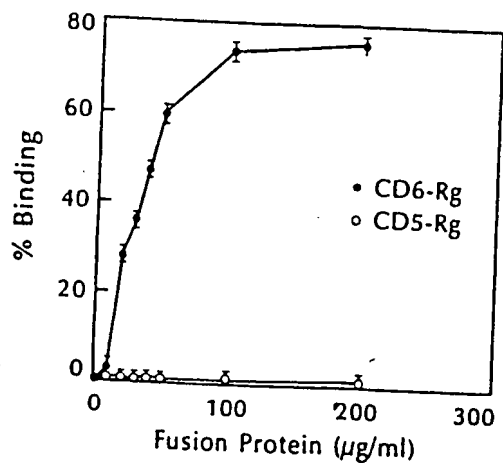
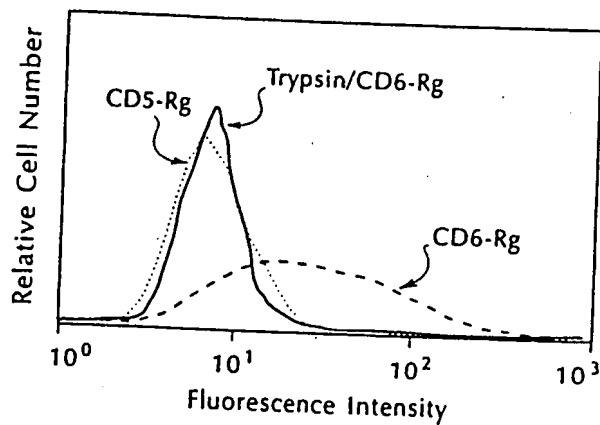
No Binding  
( $< 5\%$ )



**FIG. 8**

RECTIFIED SHEET (RULE 91)

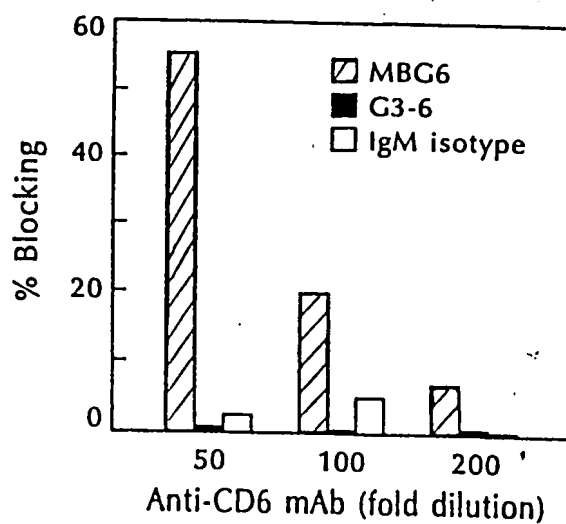
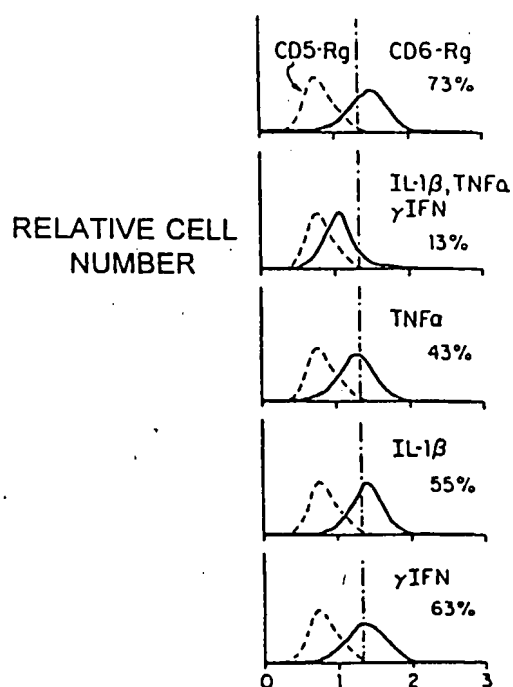
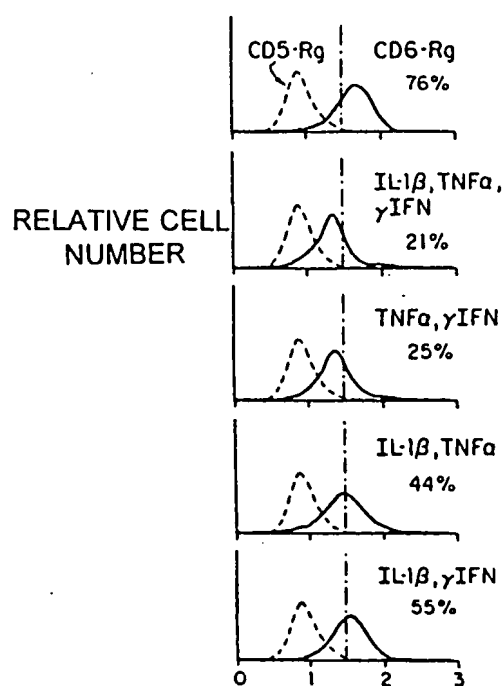
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**FIG. 10****FIG. 9A****FIG. 9B**



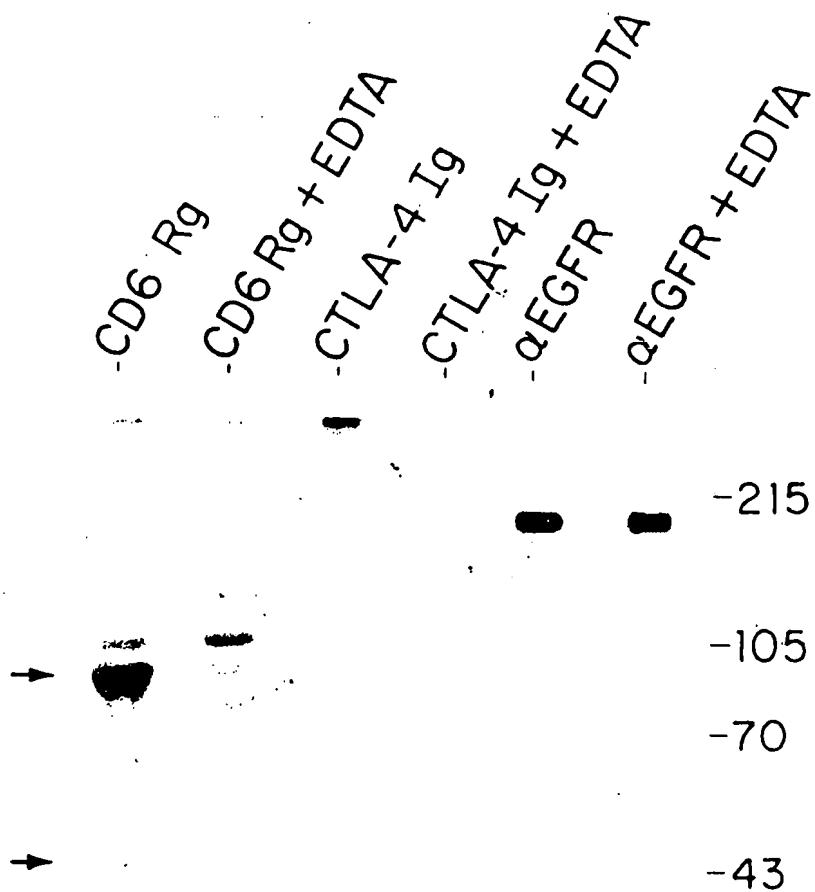
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## CHARACTERIZATION OF THE CD6 LIGAND(S)

**FIG. 11****FIG. 12A****FIG. 12B**

LOG FLUORESCENCE INTENSITY      LOG FLUORESCENCE INTENSITY

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**FIG. 13**

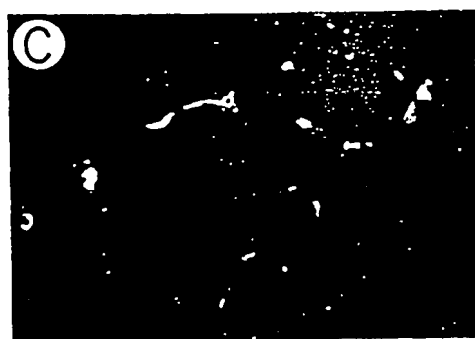
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**FIG. 14A**



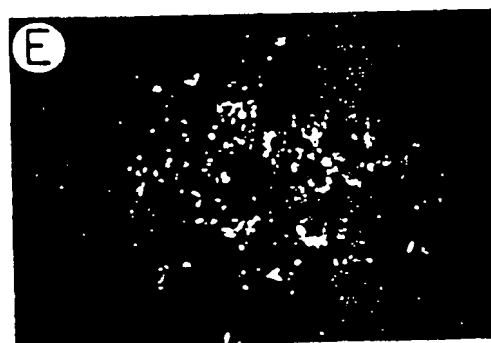
**FIG. 14B**



**FIG. 14C**



**FIG. 14D**



**FIG. 14E**



**FIG. 14F**

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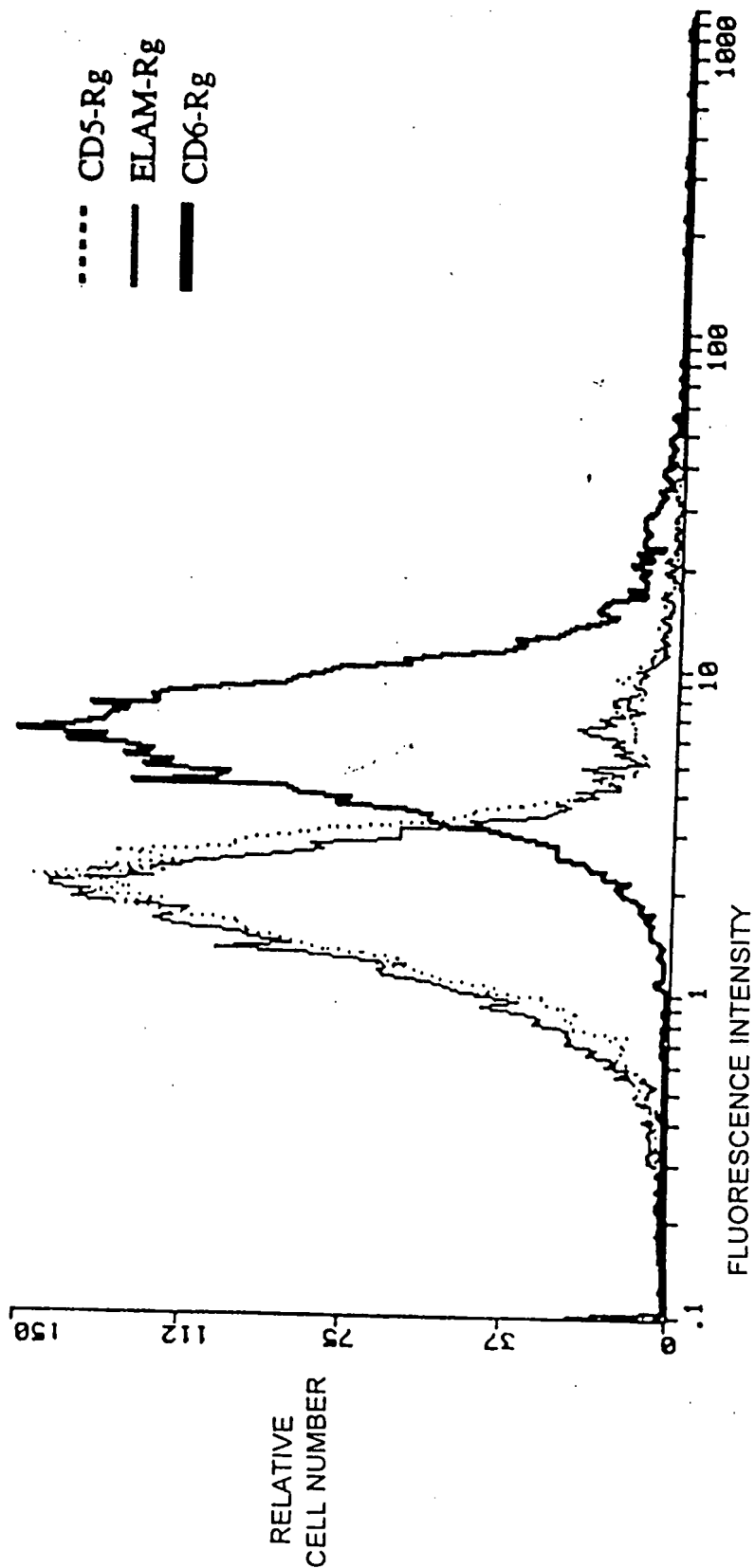
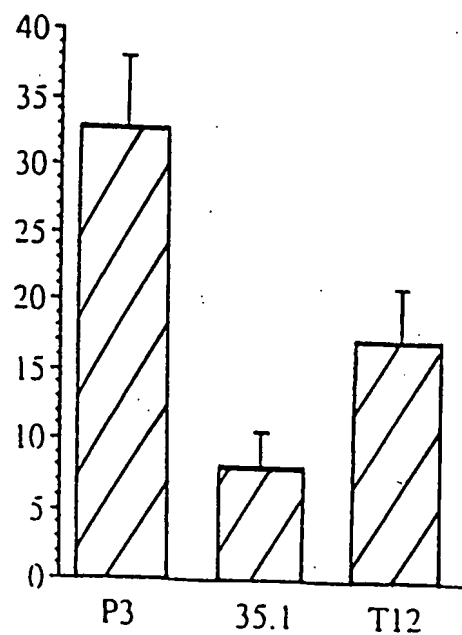
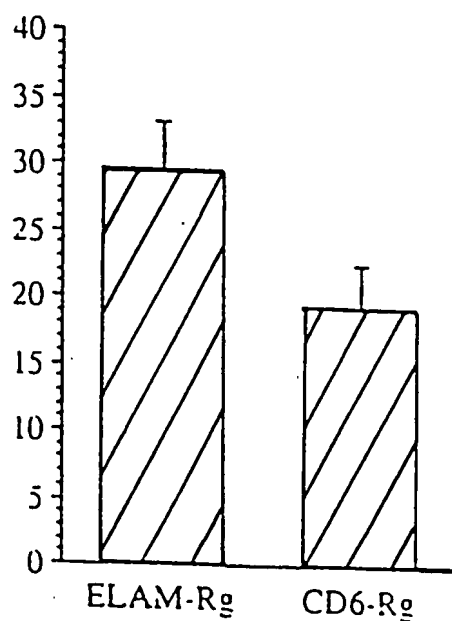


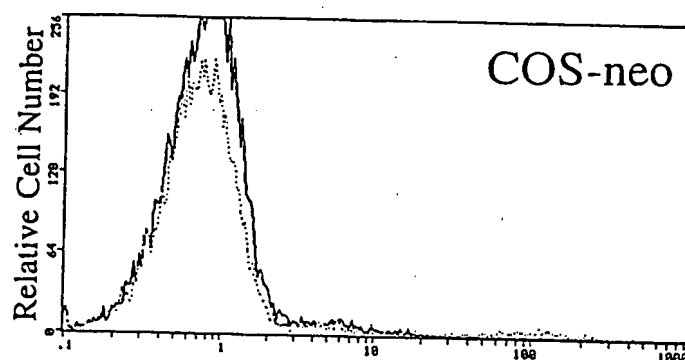
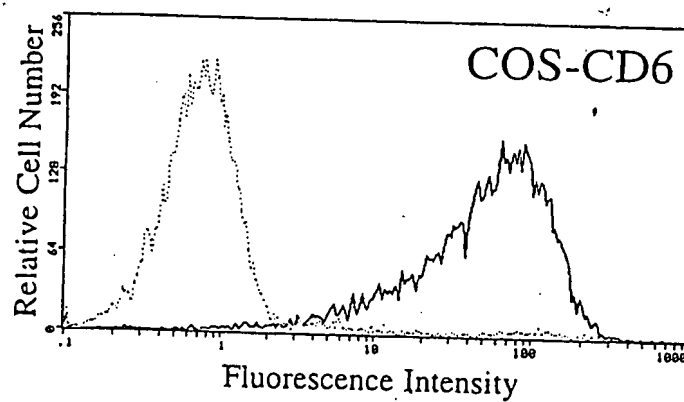
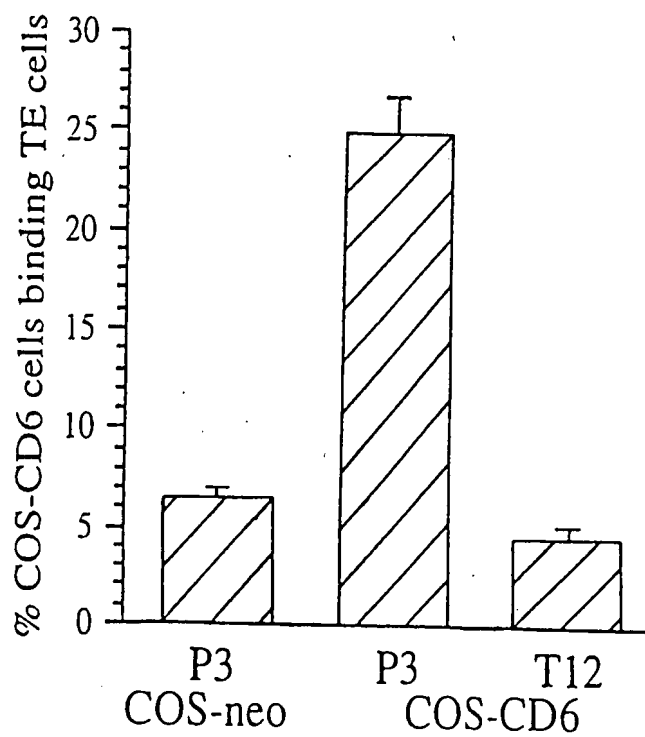
FIG. 15

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% TE CELLS BINDING  
THYMOCYTES**FIG. 16A**% TE CELLS BINDING  
THYMOCYTES**FIG. 16B**

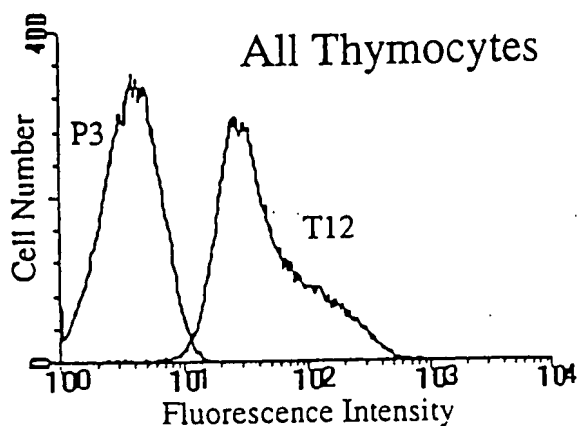
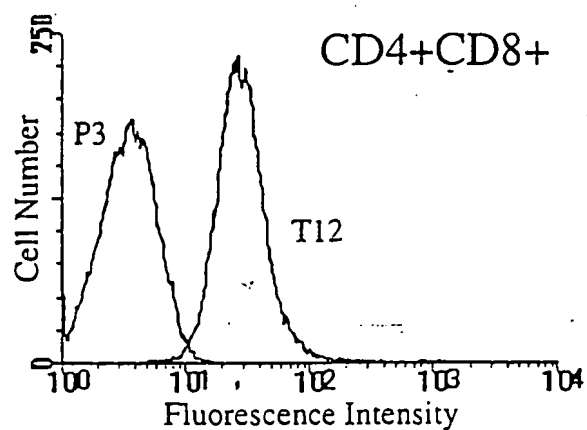
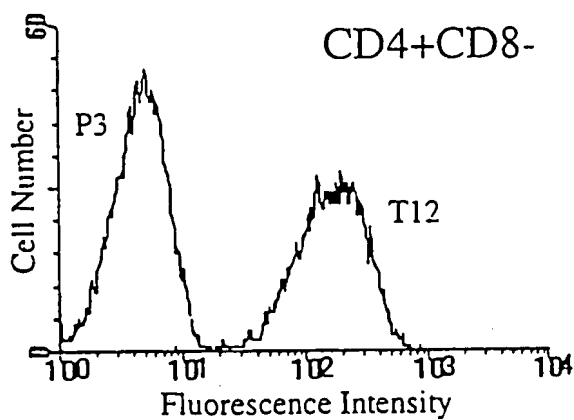
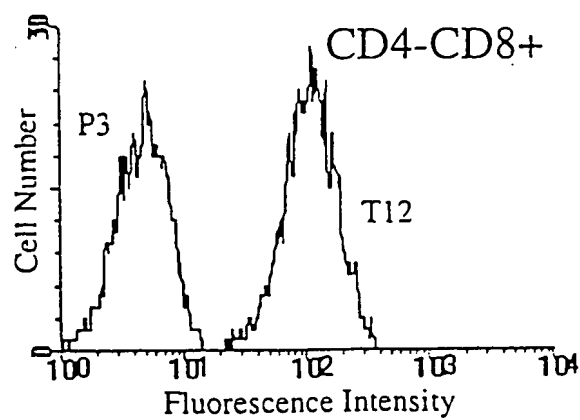
RECTIFIED SHEET (RULE 91)

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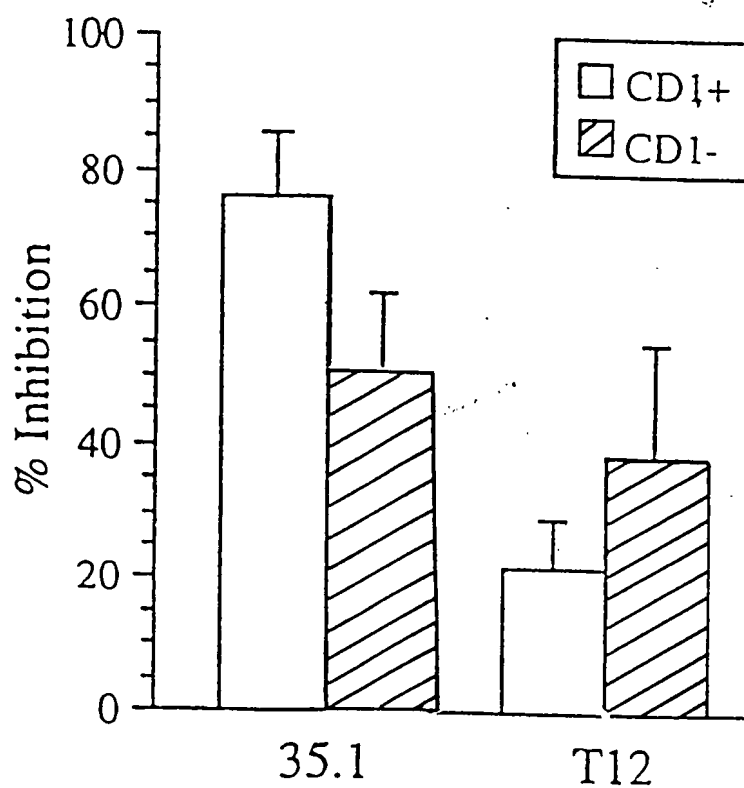
**FIG. 17A****FIG. 17B****FIG. 17C**

RECTIFIED SHEET (RULE 91)

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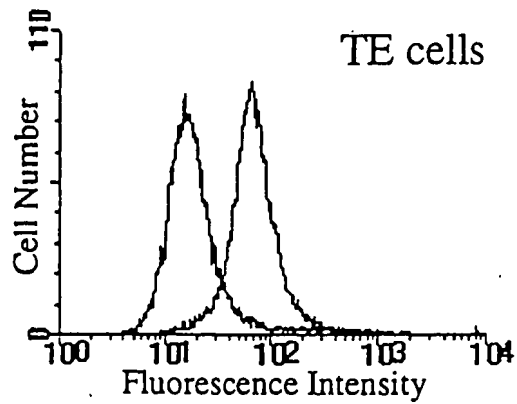
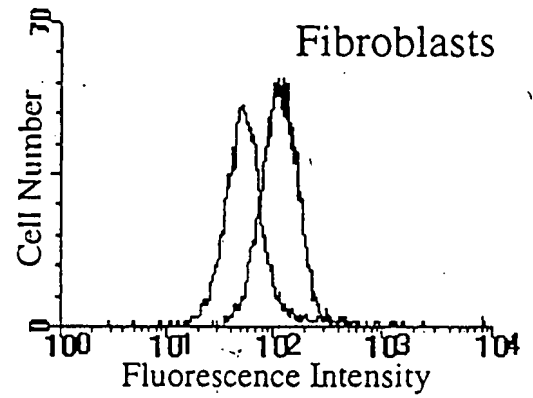
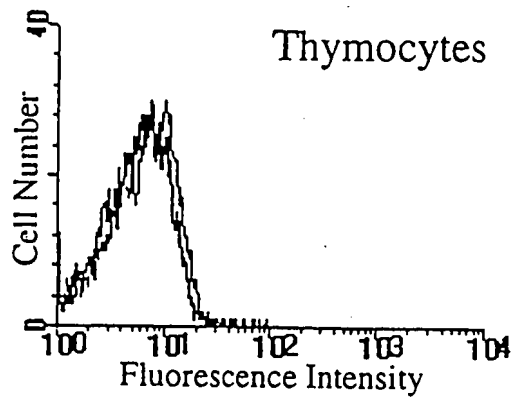
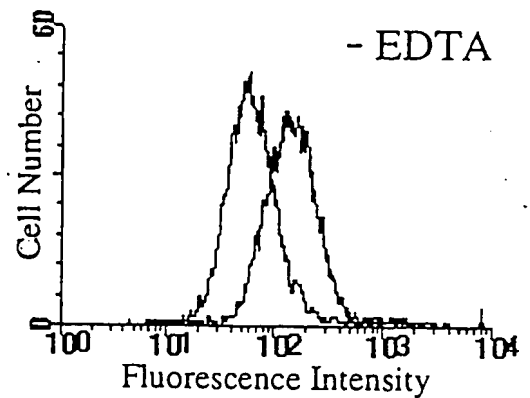
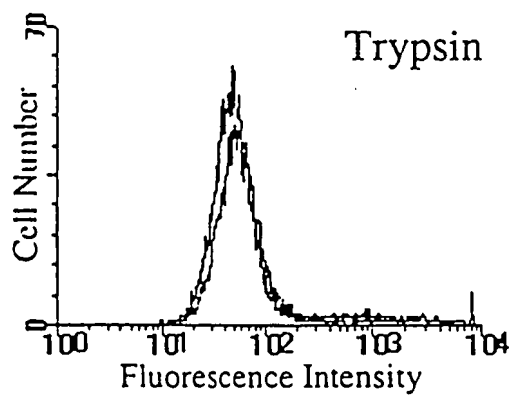
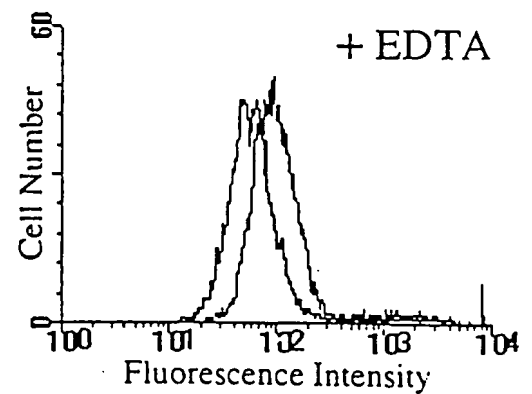
**FIG. 18A****FIG. 18B****FIG. 18C****FIG. 18D**

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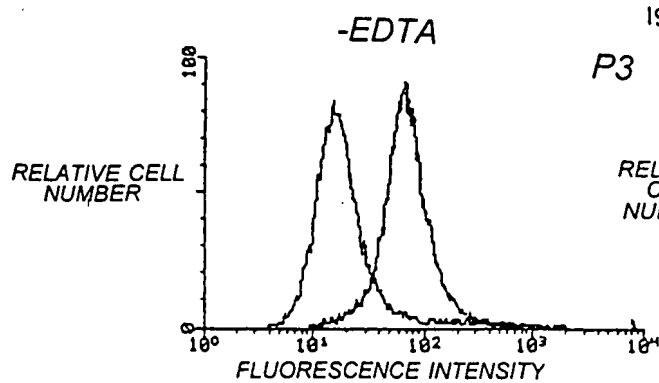
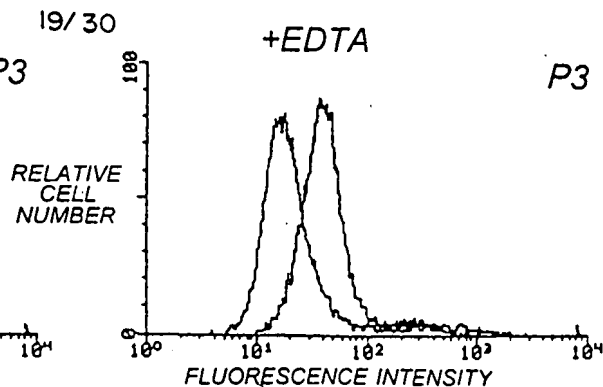
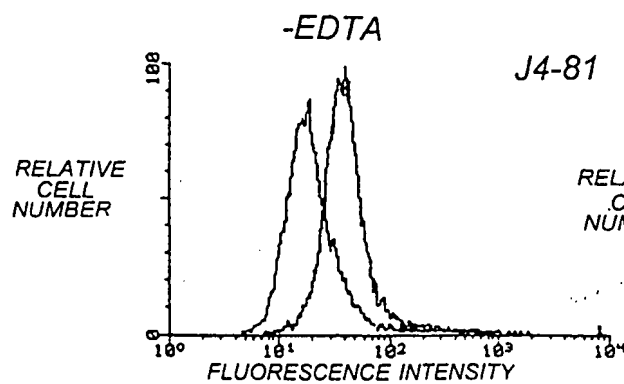
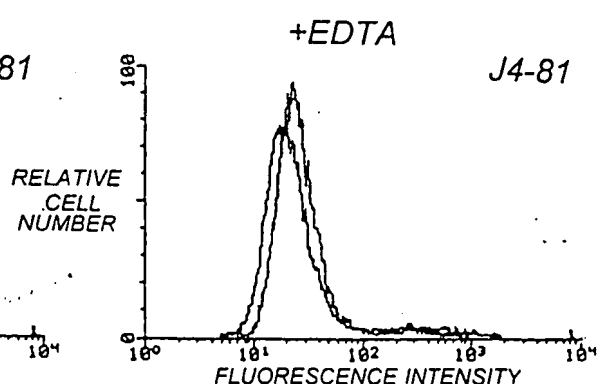
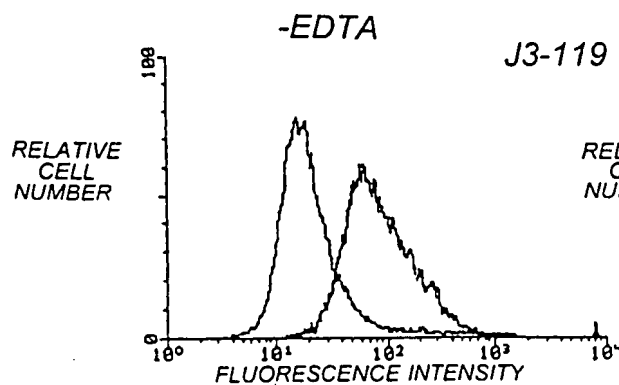
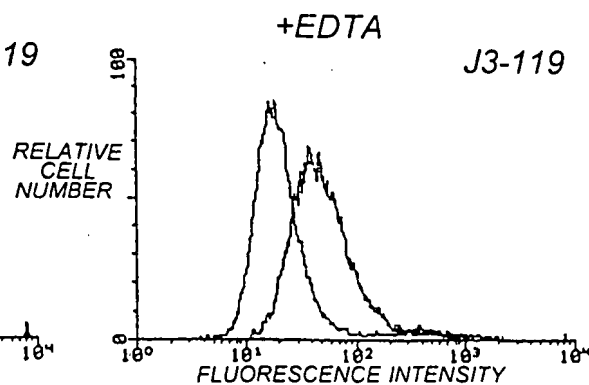
**FIG. 19**



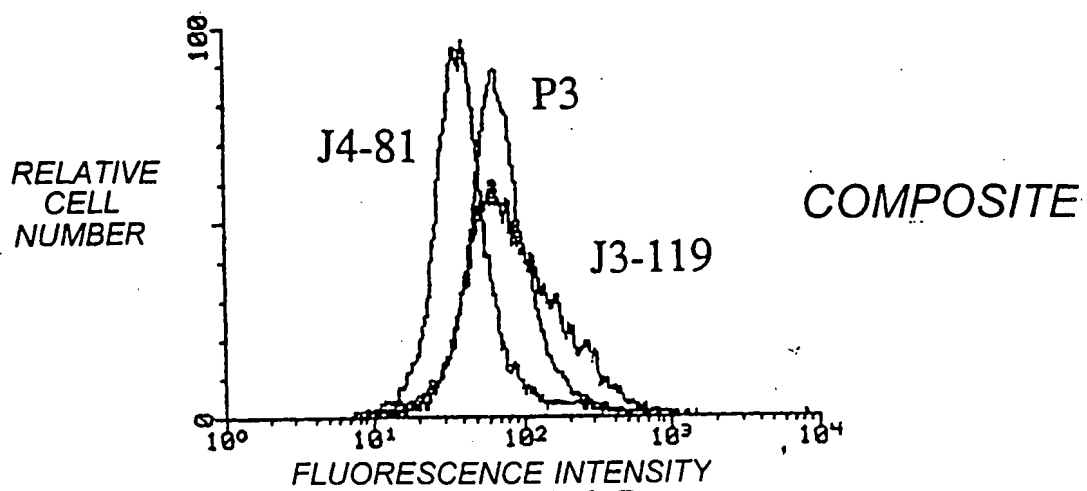
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**FIG. 20A****FIG. 20B****FIG. 20C****FIG. 20D****FIG. 20E****FIG. 20F**

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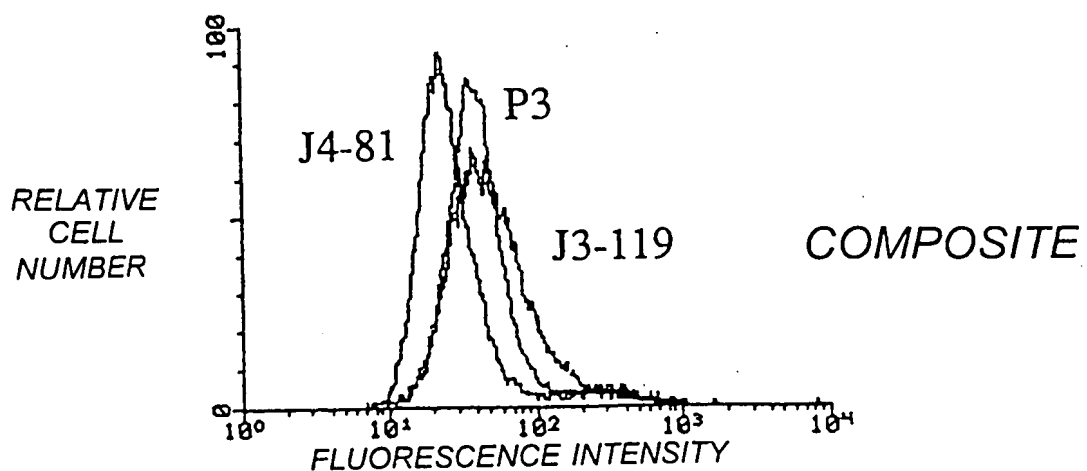
**FIG. 21A****FIG. 21B****FIG. 21C****FIG. 21D****FIG. 21E****FIG. 21F**

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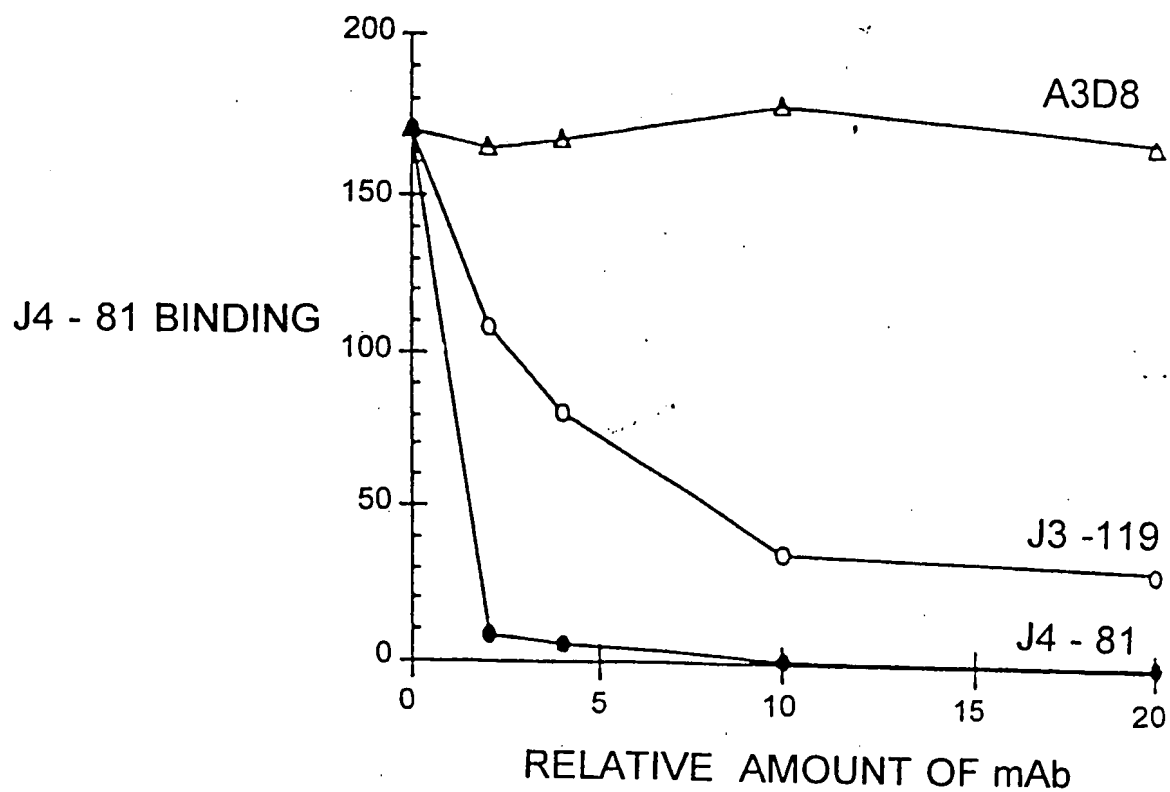
**FIG. 21G**

+EDTA

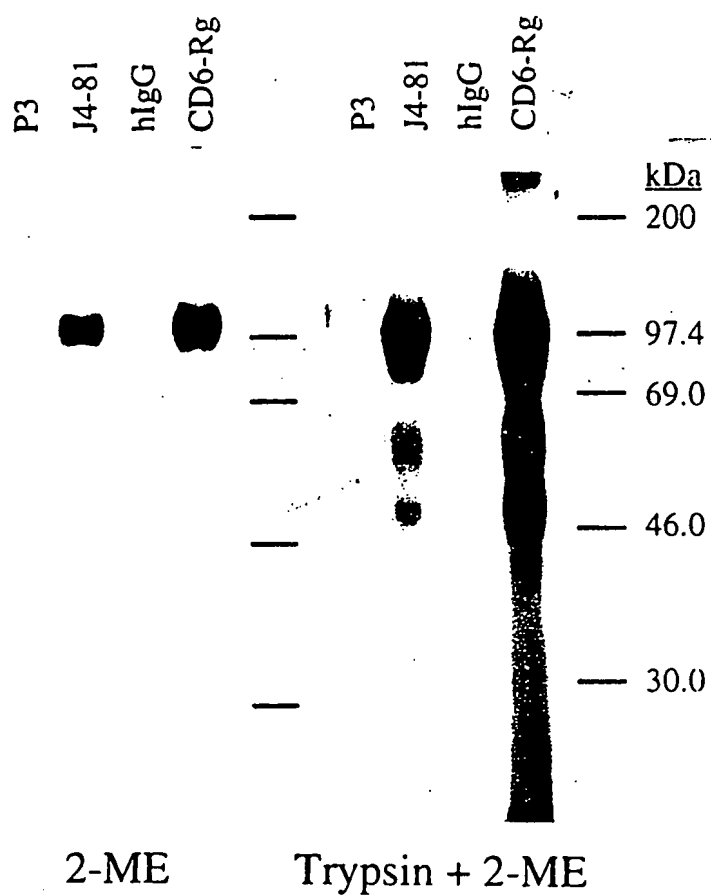


**FIG. 21H**

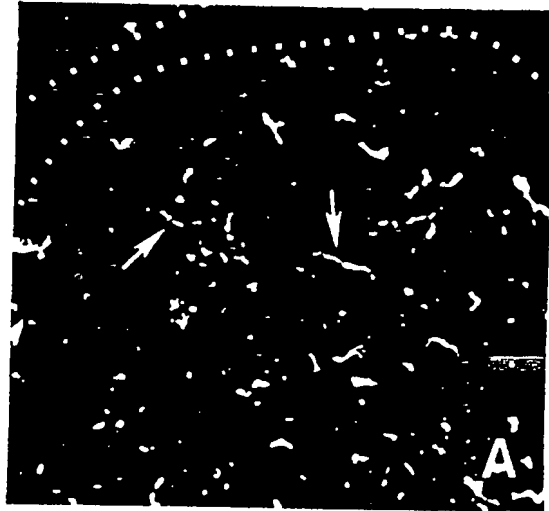
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**FIG. 22**

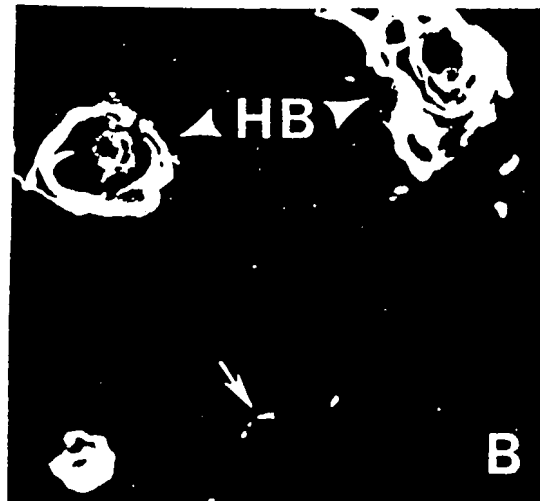
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**FIG. 23**

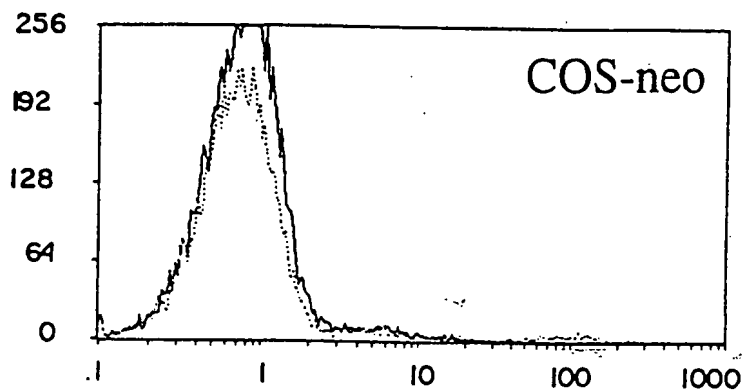
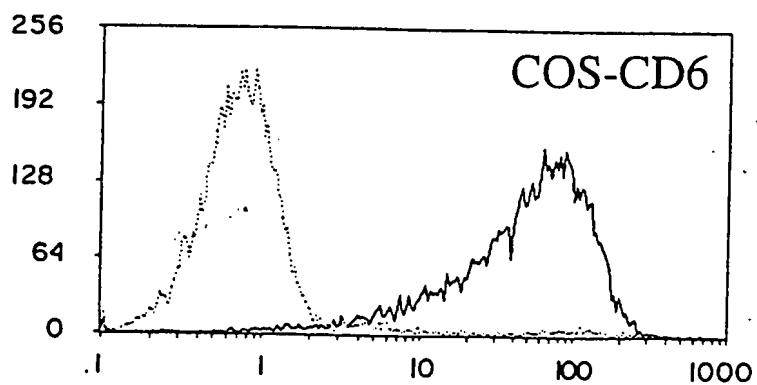
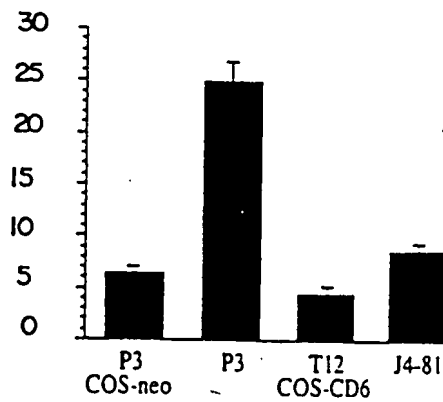


**FIG. 24 A**



**FIG. 24 B**

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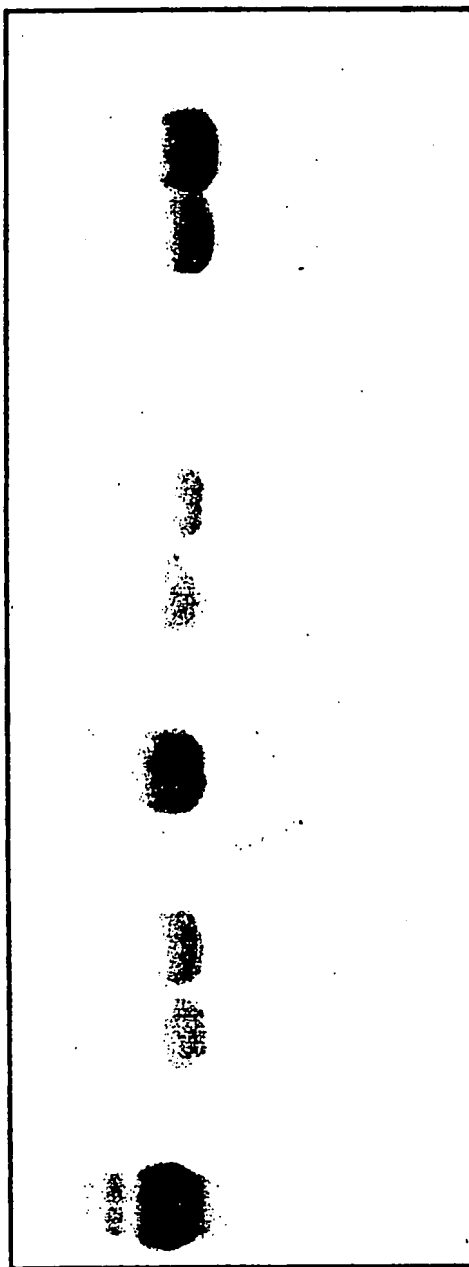
**FIG. 25A****FIG. 25B****FIG. 25C**





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Monocytes  
PBMC  
PHA B15  
CEM  
MOLT4  
RAMOS  
RAJI  
DAUDI  
YT  
K562  
U937  
HL60  
HBL100  
COS M6



9.5—  
7.5—  
4.4—  
2.4—  
1.4—

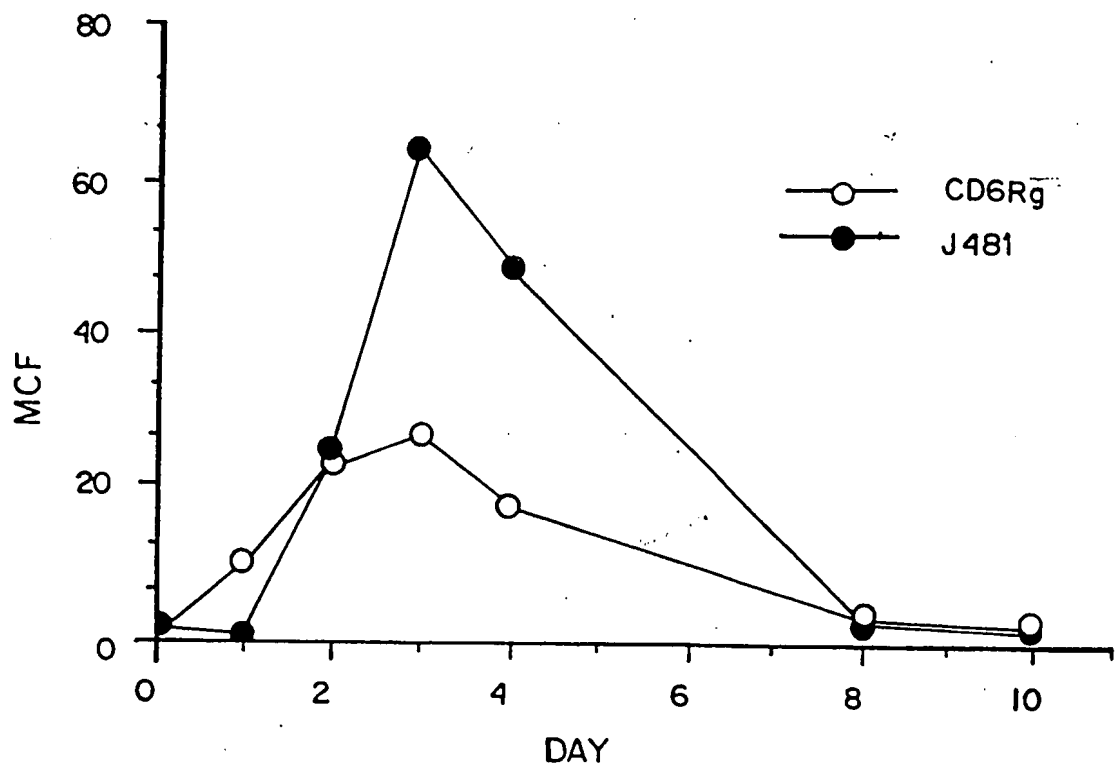
ALCAM



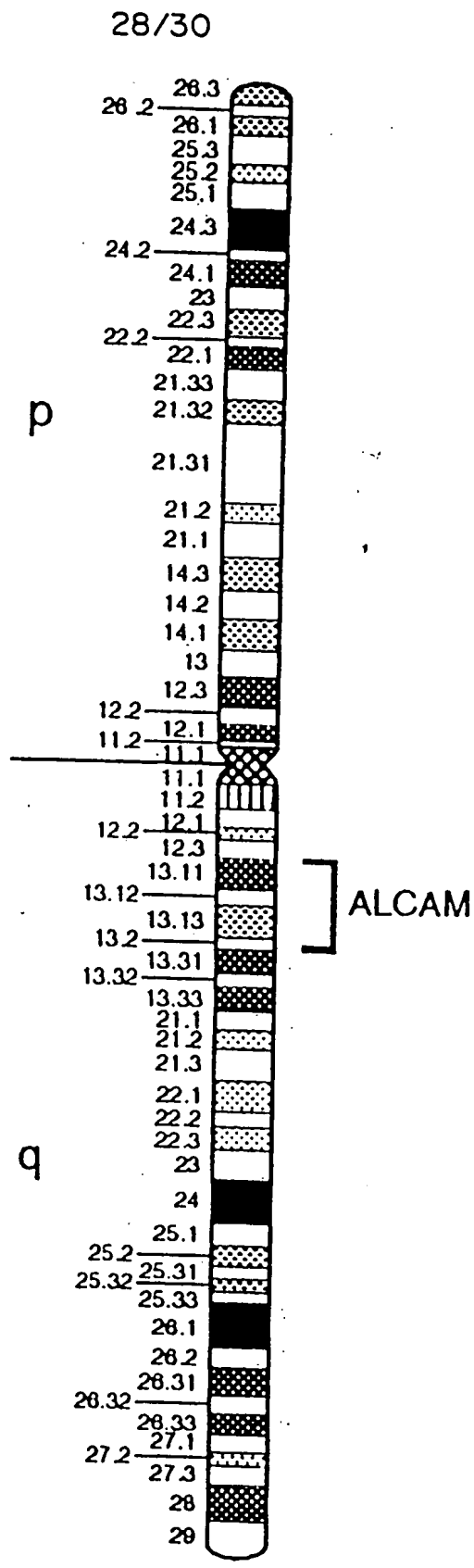
GAPDH

FIG. 26B

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**FIG. 26C**

**FIG. 27**



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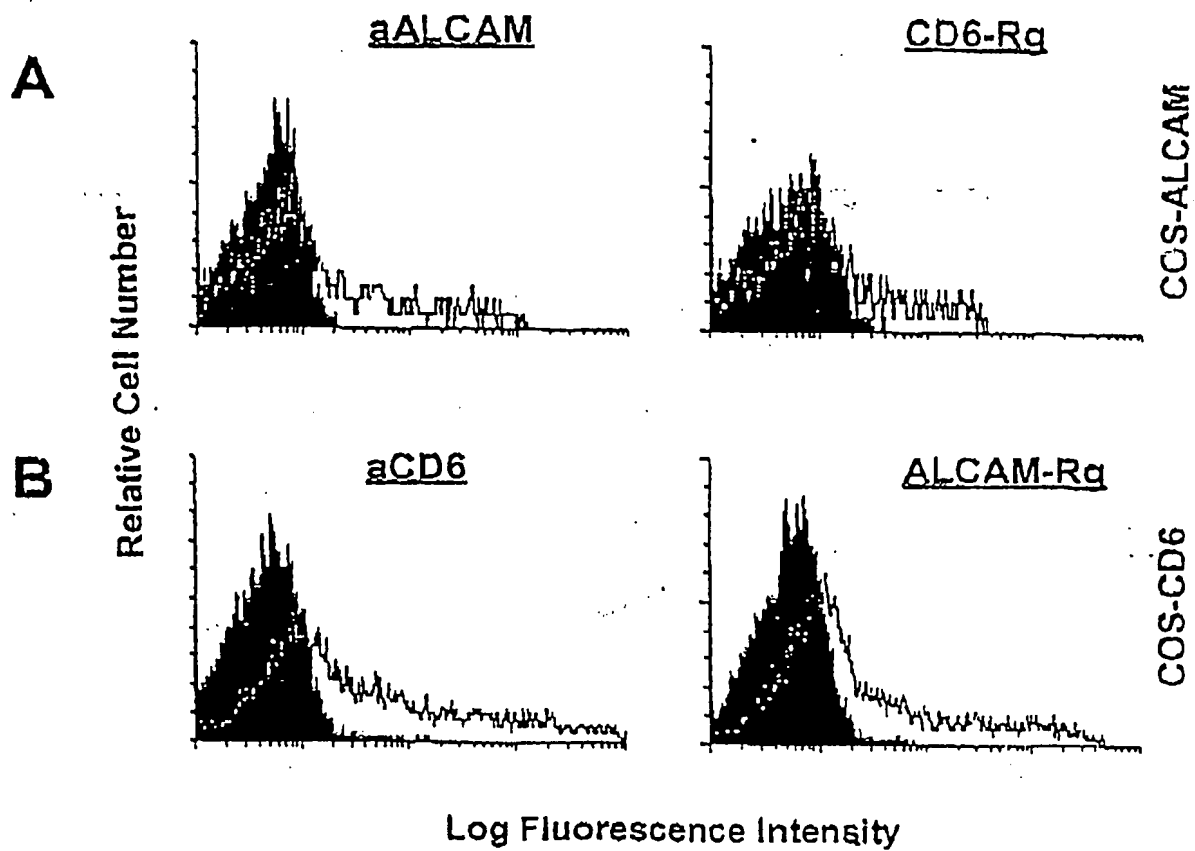


Fig 28

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[illegible]

**FIG. 29**

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/06010**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C07K 7/04, 14/47

US CL : 530/350, 395; 435/69.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 395; 435/69.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Biosis, Medline, WPI

search terms: CD6 ligand, CD6 binding protein, calcium dependent ligand.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to class. No.
P, Y	Journal of Experimental Medicine, Volume 181, issued June 1995, Bowen et al, "Cloning, Mapping, and Characterization of Activated Leukocyte-Cell Adhesion Molecule (ALCAM) a CD6 ligand", pages 2213-2220, see pages 2215-2217.	1-10
X --- Y	Journal of Experimental Medicine, Volume 181, issued April 1995, Patel et al, "Identification and Characterization of a 100-kD Ligand for CD6 on Human Thymic Epithelial Cells", pages 1563-1568, see pages 1564-1566.	1, 3-6, 9, 10 ----- 2, 7, 8



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

09 JULY 1996

Date of mailing of the international search report

23 JUL 1996

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SALLY P. TENG

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/06010

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Molecular Immunology, Volume 26, Number 11, issued 1989, Swack et al, "Structural characterization of CD6: Properties of Two Distinct Epitopes Involved in T Cell Activation", pages 1037-1049, see pages 1040-1042.	1, 2, 5, 10
X	Cellular Immunology, Volume 158, issued 1994, Wee et al, "Characterization of a CD6 Ligand(s) Expressed on Human- and Murine-Derived Cell Lines and Murine Lymphoid Tissues", pages 353-364, see pages 358-360.	1-10

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/06010

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

I-10

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/06010

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-10, drawn to CD6 ligand.

Group II, claims 11-13, drawn to mimotope of the CD6 ligand.

Group III, claims 14-16, drawn to a method of inhibiting the binding of CD6.

Group IV, claims 17-19, drawn to a method of screening test compounds capable of inhibiting binding of CD6 to CD6 ligand.

Group V, claim 20, drawn to antibodies against the CD6 ligand.

Group VI, claims 21-27, drawn to nucleic acid encoding the CD6 ligand and method of using the nucleic acid to make the ligand.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: It is noted that the expression "special technical feature" is defined in Rule 13.2 as meaning "those technical features that define a contribution which each of the inventions, considered as a whole, makes over the prior art". Group I directed to a CD6 ligand is anticipated by the prior art because the term "CD6 ligand" includes antibodies against CD6 which are well-known in the art. Therefore, Group I lacks a special technical feature. The special technical features of Groups II and V are mimotope of a CD6 ligand and antibodies specific for a CD6 ligand, which are structurally and functionally distinct compounds. The special technical feature of Group III is a method of using a CD6 ligand or mimotope to inhibit the binding of CD6 to its ligand. The special technical feature of Group IV is a method of using either CD6 or CD6 ligand to screen test compounds for its ability to inhibit CD6 to its ligand. Groups III and IV are characterized by distinct method steps, goals, and starting materials. The special technical feature of Group VI is the nucleic acid encoding the CD6 ligand. The special technical feature of each Group is not the same or does not correspond to the special technical feature of any other group because the products of Groups II and V do not share the same amino acid sequence and are structurally distinct from the product of Group VI and the methods of Groups III and IV use different reagents and method steps to perform different goals. The Groups are not linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

Group III contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows: CD6 ligand and a mimotope of the CD6 ligand. In the event that the fee for searching Group III is paid, the first named species, CD6 ligand, will be searched. An additional fee is required for a search of the mimotope.

The claims are deemed to correspond to the species listed above in the following manner:

1. CD6 ligand—Claims 14-16

2. Mimotope of the CD6 ligand—Claims 14-16

The reasons that the CD6 ligand and mimotope lack unity of invention have been set forth in the paragraph above.